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Experiments *in vitro* on the Role of Movement in the Development of Joints

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WITH ONE PLATE

IT has been pointed out by Fell & Canti (1934) as a result of their experiments *in vitro* concerning the early formation of the avian limb skeleton and knee-joint, that the *appearance* of the articular rudiment is independent of the blood- and nerve-supply as well as of mechanical influences. These authors believe that the formation of articular surfaces occurs in consequence of the differential growth of the scleroblastema (the 'Anlage' of the limb skeleton, skeletal rudiment) the essential factor in joint formation being the association of undifferentiated tissue with the rapidly growing chondrification centres. They emphasize, however, that only the earlier stages of joint formation can be obtained *in vitro*; the conditions of cultivation are not adequate for the further development of the joints. The articular rudiment disappears by secondary fusion of the cartilages of the limb skeleton. Fell & Canti mention the possibility that though mechanical movement is not necessary for the *appearance* of the joint, it may be an important and possibly an essential factor in its subsequent development.

Gündisch (1943), examining cultures of chick-limb scleroblastemas, came to the conclusion that the growth observed *in vitro* is, for the most part, erroneously interpreted as organotypical, since the development *in vitro* of the organ anlage represents a histotypical proliferation of tissues. According to his work only the cartilage cells continue to differentiate in the explanted limb skeletal blastema, multiplying and producing ground substance. Thus cartilage centres are produced which should be considered as separate cultures of cartilage tissue in an organ culture undergoing dedifferentiation. In consequence of the histotypical growth of these cartilage centres the loose tissue between the articular surfaces becomes chondrified. The more developed the joints are at explantation, that is, the smaller the undifferentiated tissue between the articular ends is, the later the chondrification takes place. Gündisch mentions that movement inhibited to some extent the fusion of the cartilaginous surfaces.

In the present experiments the influence of mechanical movement on the

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development *in vitro* of the knee-joint of 6–7-day-old chick-embryos was studied. At this stage the joint has already appeared. No consideration has been given to the earlier stages of its development.

MATERIAL AND METHOD

Knee-joints of 6–7-day-old chick-embryos were cleaned from soft tissues, care being taken that the perichondrium should remain intact. The joints were cultivated, some according to Fell's watch-glass technique, some in Carrel flasks. Always the two limbs of the same embryo were explanted in the same watch-glass or flask, one limb being subjected to movement, the other one serving as control. The limbs were moved with a thin glass rod, five times a day. A mixture of fowl blood-plasma and fowl embryonic extract (2 : 1) was employed as medium. The explants cultivated on watch-glasses were transferred to new medium every 48 hours. The liquid medium in the Carrel flasks, composed of Tyrode solution and embryonic extract (5 : 1), was changed every two days. Forty joints were explanted. After cultivation for different periods of time the cultures were fixed in Susa mixture, embedded in paraffin, and cut in serial sections. The sections were stained with haemalum-eosin, toluidine blue, or Azan.

RESULTS

It was observed that both the unmoved controls and the joints subjected to movement presented some regressive alterations which, especially in certain portions of the epiphyseal cartilage, were manifested in a pale staining of the nuclei of the cartilage cells and in disintegration of the ground substance. Besides this regression, however, there were to be found tissue differentiations too, such as hypertrophy of cartilage cells in the diaphysis and formation of a perichondral bony sheath, as well as proliferation of cartilage tissue originating from the epiphyseal cartilage. *In explants which had not been moved* this proliferation of the cartilage tissue often forms a continuous cartilage bridge between the femur and tibia by chondrifying the undifferentiated interarticular tissue (Plate, fig. 1). There is no remainder of the interarticular tissue, the site of fusion of the cartilage-anlage being marked only by the elongated cartilage cells that correspond to the previously free surface (Plate, fig. 2). There is a little basophil ground substance between the cells which completely masks the collagen fibres. In other cases there is an entirely irregular histotypical proliferation of epiphyseal cartilage which tends to fusion.

In explants that were moved the cartilaginous fusion of the parts of the limb skeleton does not take place at all. Between the articular ends there is to be seen a loose mesenchymal tissue with its cells situated along the articular surfaces (Plate, fig. 3). The flat cells are arranged in 4–5 rows with collagen fibres between them. Beginning from the 6th row the fibres are masked by the ground substance and here the cells assume a more rounded form. The superficial layers show the character of procartilage (Plate, fig. 4). More striking still is the separation of

the articular surfaces with the formation of an articular cavity in the explant shown in Plate, fig. 5 (knee-joint of a 7-day-old chick-embryo cultivated for 6 days, moved five times a day). The upper part of the picture is particularly interesting in that the proliferating young cartilage there shows a definite articular surface. The cells of the articular surfaces are flattened and show the character of a procartilage. Collagen fibres are to be found only between the cells of the most superficial 2–3 rows. The amount of ground substance rapidly increases in the deeper layers. The arrangement of the flattened cells is parallel to the articular surface. There is no remainder of tissue between the articular surfaces opposite to each other (Plate, fig. 6).

In summary, of 20 moved explants, 7 showed complete separation of femur and tibia with formation of an articular cavity; and 13 had well-formed articular surfaces but with loose tissue between them, though no cartilaginous fusion. Of 20 control (unmoved) explants, 9 showed complete cartilaginous fusion between femur and tibia, and 11 showed entirely irregular histotypical proliferation of the epiphyses.

DISCUSSION

Székely (1943) demonstrated with limbs of human embryos that muscular activity may be presumed—judging on morphological grounds—to occur as early as the cartilaginous skeletal rudiments appear. At first the muscular activity, by forming the tendon structure, produces only a very slight movement. Later, with the strengthening of muscles and tendons, a movement with greater amplitude of flexion and extension becomes possible, which promotes the formation of the articular surfaces and capsule and prevents at the same time the fusion of the articular ends.

Hamburger (1929) denied the role of movement in the formation of joints. He extirpated in frogs—in the neural-plate stage—the lumbosacral part of the spinal cord. After the operation the morphogenesis of the reduced limbs was stated by him to be normal. As a counter-argument it may, however, be mentioned that since the paralysed limbs were floating in water the articulations had some possibility of movement which might have provided the minimal stimulus necessary for the development of a joint. The articular cavity actually obtained was in fact reduced.

The experiments recorded here on the role of movement in the development of joints suggest the following points:

1. In explanted joints which are not moved during cultivation cartilaginous fusion across the joint occurs, as observed by several authors (Gündisch, 1943, *in vitro*; Pellegrini, 1934, in chorio-allantoic grafts) and in my own control preparations. This fusion is prevented if movement, resembling the normal movement at the joint, is produced during cultivation.

2. Not only is fusion prevented by movement, but the formation of an articular cavity is induced by movement.

3. Movement also exerts a formative effect on the shape and structure of the articular surfaces.

It may therefore be suggested that proper differentiation of a joint depends on its undergoing movement.

SUMMARY

1. The effect on joint differentiation of movement at the knee-joint of leg rudiments of 6–7-day-old chick-embryos cultivated *in vitro* has been studied.

2. In control explantations, where no movement was applied, cartilaginous fusion across the joint of the skeletal parts was generally found. Movement prevents this fusion. An articular cavity was sometimes formed in the moved explants but not in the controls.

3. Movement exerts a formative effect on the shape and structure of the articular surfaces, and on the histotypical proliferation of cartilage tissue.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATE

All microphotographs were taken of sections stained with Azan (Heidenhain).

Fig. 1. Knee-joint of 7-day-old chick-embryo cultivated for 6 days, not moved. Cartilaginous fusion of femur and tibia. ×40.

Fig. 2. Cartilage bridge between femur and tibia, from the preparation seen in fig. 1; higher magnification. ×400.

Fig. 3. Knee-joint of 7-day-old chick-embryo cultivated for 6 days, moved five times a day. Definite articular surfaces with loosened tissue between them. ×40.

Fig. 4. Area between articular surfaces from the preparation seen in fig. 3; at greater magnification. ×200.

Fig. 5. Knee-joint of 7-day-old chick-embryo, cultivated for 6 days, moved five times a day. Articular surfaces well developed, articular cavity present. ×40.

Fig. 6. Articular surfaces from the preparation seen in fig. 5 with greater magnification. ×400.

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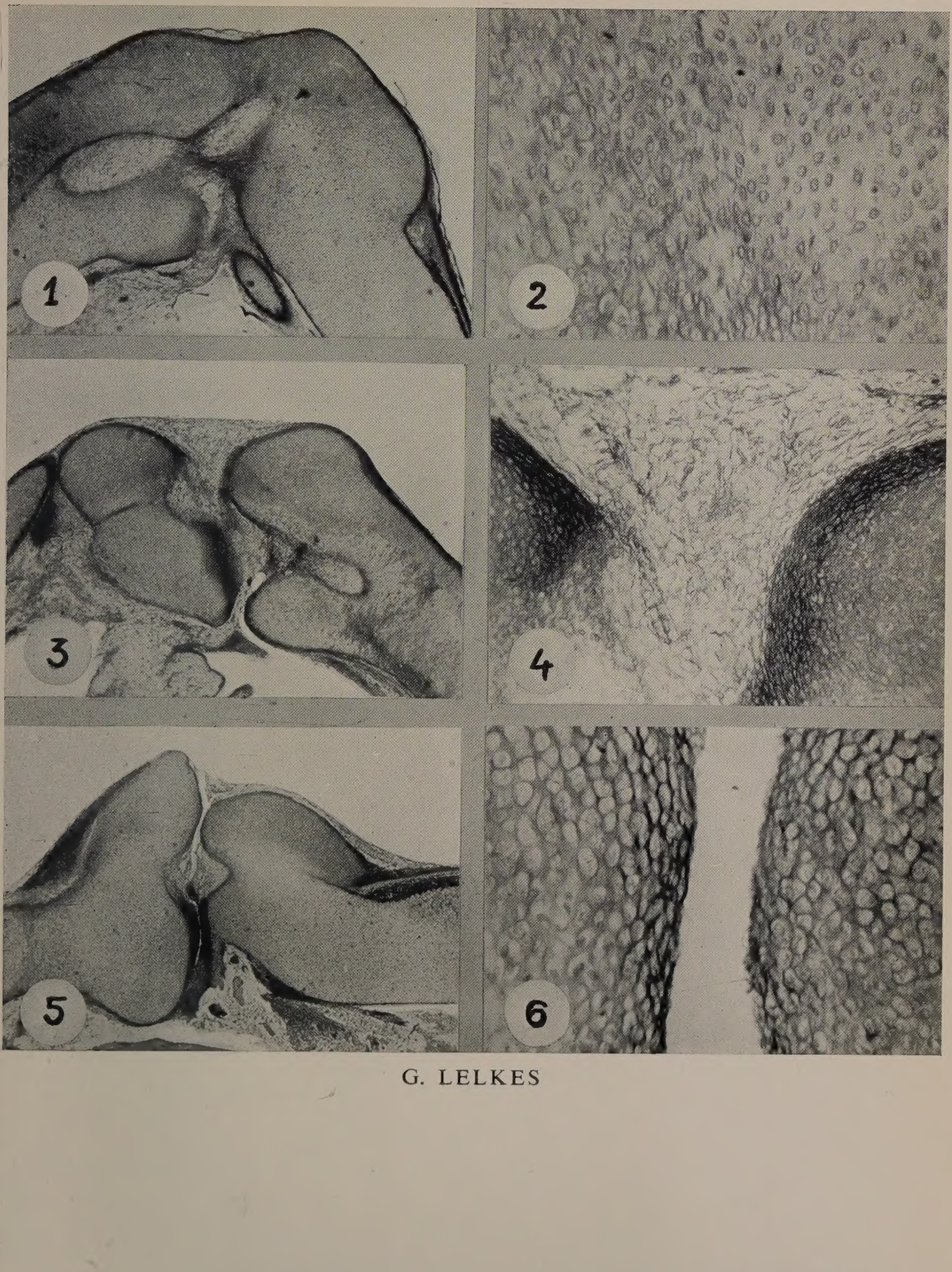

G. LELKES

Further Observations on the 'Metaplasia' of an Amoeba, *Naegleria gruberi*

by E. N. WILLMER[1]

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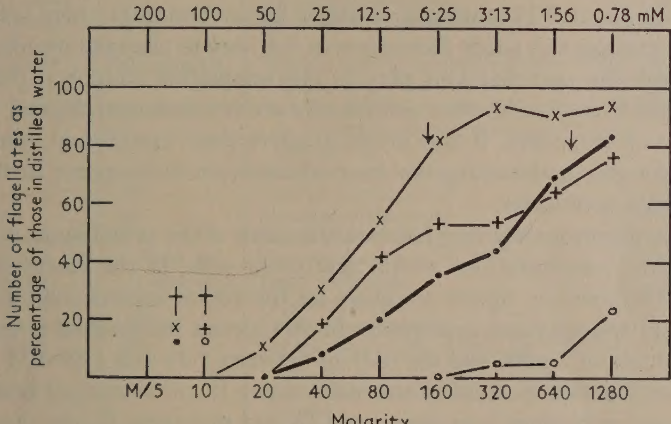
THE development of flagella by *Naegleria gruberi*, which occurs when a culture of organisms in the amoeboid form is treated with distilled water, has been shown (Willmer, 1956) to be a phenomenon probably related more to the ionic



TEXT-FIG. 1. Action of various kations on the numbers of amoebae which become flagellate as percentage of the number becoming flagellate in distilled water [=100].

Ordinates: percentage which become flagellate in experimental solution. (Number in distilled water = 100.)

Abscissae: molar concentrations of solutions.

The arrows represent the K and Na content of *Spirostomum* as determined by Carter (1957). The crosses with appropriate symbols represent the concentrations at which the amoeboid form is damaged, and rounds off.

•——• NaCl +——+ $CaCl_2$ ×——× KCl o——o $MgCl_2$

balance between the organism and its external environment than to the simple movement of water in and out of the cell in response to total osmotic changes. From Text-fig. 1 it may be seen that with respect to the response of the amoeba to different concentrations of the various salts investigated there are for each

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salt two somewhat critical concentrations. In the case of NaCl solutions in distilled water these turning points occur at about 30 mM. and 1 mM., and in the case of KCl solutions at about 50 mM. and 6 mM. Above the higher of these concentrations in each case the organisms always assume the amoeboid form, and below the lower they become flagellate at least as frequently as they do in distilled water.

As with some other protozoa (e.g. *Paramecium* (Hayes, 1930); *Spirostomum* (Carter, 1957)) there may well be some concentration of cations within the cell which can be considered as an equilibrium concentration and which can be maintained with the minimum expenditure of energy or which is, for some other reason, 'preferred' by the cell. This concentration may not be rigidly fixed but may perhaps vary to a limited extent according to the external conditions. Indeed, in some other species of amoebae (e.g. *Amoeba* (*Mayorella*) *lacerata* (Hopkins, 1946)), in *Paramecium* (Kamada, 1935, 1936) and in the peritrich ciliate *Vorticella* (Mast & Bowen, 1944), which maintains an osmotic pressure difference of about 0·33 atmospheres above its surroundings, there is evidence that the organisms can adapt themselves in this way to changed osmotic conditions, though the part that ions play in this adaptation cannot at present be distinguished from that of other osmotically active substances. Sooner or later, however, such organisms, if they are to preserve their equilibrium, must adopt means of forcibly maintaining the internal concentration against further gain or loss of ions or of water.

There are unfortunately very few measurements of the actual ionic, as distinct from osmotic, concentrations within protozoan cells. In the recent paper by Carter (1957), however, figures are given for the potassium and sodium content of the ciliate *Spirostomum ambiguum*. In this species the internal potassium is regulated at about 7 mM. and the sodium between 1·01 and 1·49 mM. in spite of an almost fourfold increase in external sodium. It will be noticed first that, in *Spirostomum*, potassium is in excess and second that these figures correspond approximately to the lower figures for K^+ and Na^+ mentioned above for *Naegleria* at which these amoebae tend to assume the flagellate form. In some preliminary estimations by flame photometry of the sodium and potassium of *Naegleria* itself, carried out in collaboration with Dr. Whittam, the sodium content has been found to be higher than the potassium content in a ratio of about 4 : 1. The absolute concentrations are, however, at present uncertain. The estimates indicate that they may be considerably higher than those for *Spirostomum*, but the actual quantities being measured by the methods employed may be different in the two cases and so the figures may not be strictly comparable; for the present then the issue must remain undecided.

On the basis of the results shown in Text-fig. 1 it could be suggested with some justification that *Naegleria* assumes the amoeboid form when the external cation concentration exceeds the internal, and the flagellate form when the situation is reversed. The flagellate form may indeed be an adaptation towards cation

conservation, while in the amoeboid form the cell is more concerned with preventing the entry of cations. Water movements in the opposite direction may, of course, also be involved; i.e. the flagellate form needs to eject water while the amoeboid form may need to conserve it. In both forms there is a contractile vacuole.

There are many estimations, by a variety of methods, of the total osmotic concentration of the cytoplasm of different freshwater protozoa, though these are obviously not all relevant to the particular problem, for the internal osmotic concentration may be maintained not only by salts, but also by sugars, fatty-acids, amino-acids, proteins, &c. Nevertheless, from Table 1 it is clear that most of the figures for the osmotic concentration lie within, or only just outside, the critical range of concentrations mentioned above for *Naegleria*. Among them there are wide variations in the estimates, some of which may be due to the different methods employed which measure different things, some to technical difficulties in measuring such important things as, for example, the volume of an amoeba. If, however, the series of figures obtained by Gelfan (1928) for the

TABLE 1

Organism	*Internal concentration (m.osmols/l.)*	*Method*	*Reference*
Chaos chaos	94	Vapour pressure determination	Løvtrup & Pigon (1951)
Amoeba proteus	20	Electrical conductivity	Gelfan (1928)
Chaos diffluens	5	Size changes in lactose solutions	Mast & Fowler (1935)
*Amoeba mira**	200 (approx.)	Vacuolar activity suddenly increases in dilutions of sea-water below 20 per cent.	Mast & Hopkins (1941)
*Amoeba lacerata**	200 (approx.)	Vacuolar activity becomes almost constant in concentrations of sea-water above 20 per cent.	Hopkins (1946)
Stentor	70	Electrical conductivity	Gelfan (1928)
Euplotes	75	Electrical conductivity	Gelfan (1928)
Oxytricha	100	Electrical conductivity	Gelfan (1928)
Spirostomum teres	77	Electrical conductivity	Gelfan (1928)
S. ambiguum	7 mM. K	Tracer technique	Carter (1957)
S. ambiguum	1 mM. Na	Tracer technique	Carter (1957)
S. ambiguum	52	Vapour pressure	Picken (1936)
Paramecium	120	Electrical conductivity	Gelfan (1928)
Paramecium	62	Contractile vacuole activity (in 25 mM. NaCl)	Kamada (1936)
Paramecium	50	Contractile vacuole activity (in distilled water)	Kamada (1936)
Paramecium	48	Minimal O_2 consumption	Hayes (1930)
Frontonia	106	Electrical conductivity	Gelfan (1928)
Vorticella	12	Size changes in lactose solution	Mast & Bowen (1944)
Vorticella	50	Swelling of cyanide-treated organism	Kitching (1938)

* These two species can both adapt themselves to fresh water or sea-water.

electrical conductivity are studied, it will be seen that *Amoeba proteus* has a much lower conductivity than any of the ciliates. If, therefore, the optimal internal ionic concentration of amoebae in general is initially low, the organism, in order to preserve this low concentration, is likely to be more often concerned with ejecting or preventing the entry of incoming salts than with concentrating them. If that of the ciliates is initially higher, then these organisms must normally be concerned with preventing the loss of ions, with actively accumulating ions from the surroundings or with the elimination of water drawn in by the higher osmotic pressure. As mentioned above, both *Paramecium* (Kamada, 1936) and *Vorticella* (Mast & Bowen, 1944), appear to be able to maintain a constant excess of internal concentration over the external concentration in spite of quite large changes in the latter.

So far as they go, therefore, these figures are all consistent with the idea already suggested that the flagellate form of *Naegleria* is concerned with ionic conservation and the amoeboid form with preventing the accumulation of ions. In *Naegleria*, for example, water appears to be always passing into the cell in all concentrations of NaCl in the external medium up to approximately 200 mM., for it is not until this value is reached that the contractile vacuole ceases to function (Wolff, 1927). Much the same is true for *Amoeba* (*Mayorella*) *lacerata* (Hopkins, 1946). In other freshwater species the water intake, or its production by metabolism, is connected with cell movement (Hogue, 1923) and the contractile vacuole may go on ejecting water when the organism is subjected to external media which are probably distinctly hypertonic to the internal contents of the cell.

With these considerations in mind, the experiments to be described in this paper have been designed to investigate the truth or otherwise of the hypothesis that the flagellate form of *Naegleria* has an inwardly-directed cation pump while the amoeboid form has an outwardly directed pump, and that the organism can turn on the one or the other in order to maintain its ionic content within certain limits, more or less independently of the external medium.

Some of the various substances known to alter the rate of sodium or cation transport in other situations, either directly or indirectly, have been examined for their action on the behaviour of *Naegleria*. Some attention has also been paid to the relative importance of sodium and potassium, and the available evidence indicates that in *Naegleria* the one may substitute for the other, though this does not mean that the amoeba has no 'preference' for the one or the other, when both are available.

METHODS

The methods employed have, in general, been similar to those described in connexion with the earlier series of experiments (Willmer, 1956). All observations have been made by counting the flagellate cells in a single circuit of standard-sized (0·01 ml.) drops plated out on a microscope slide, four at a time, and

examined under a $\frac{2}{3}$ in. lens always keeping the edge of the drop in the field. All test solutions have been used in 0·5 ml. aliquots contained in thoroughly cleaned test-tubes and each inoculated with a single drop from a well-stirred suspension of amoebae previously washed as free from bacteria as possible (see below). Counts of those in the flagellate form have been made either on amoebae inoculated into serial dilutions (generally $\times 2$) of the substance under investigation or into quadruplicate series of tubes containing the experimental media at particular concentrations.

The use of glass-distilled water, by itself, as a standard medium, for evoking the flagellate form, has been largely abandoned in favour of an extremely dilute phosphate buffer solution, and this change has led to much more uniform results. The buffer solution (B_2) was made up so that the final concentrations were as follows:

0·2 mM. KCl
0·2 mM. $NaH_2PO_4 . 2H_2O$
0·2 mM. $Na_2HPO_4 . 12H_2O$

This solution has the advantage of preventing large pH changes in response to such random variables as small changes in CO_2 content, and yet its salt content is not sufficient to cause any appreciable lessening of the numbers of amoebae which initially become flagellate when transferred to it, though, as will be seen, it probably hastens their ultimate return to the amoeboid condition.

In some experiments in which only the sodium ion or the potassium ion was required in the medium, the appropriate changes were made in this basic solution.

In the previous paper the rate at which the flagellate cells appeared after the amoebae were placed in experimental media was briefly discussed. In the buffer solution (B_2) the flagellate forms generally appear after about 2 hours, and the numbers increase for about another 3–4 hours, after which they remain more or less stationary for a time and then decline. This observation which is consistent with the view that the flagellate form is concerned with picking up Na^+ (or other positive ion) has led to the practice of following the time-course of most experiments instead of making counts only at one time, e.g. after 4–6 hours; this in its turn has led to a better appreciation of the behaviour of the amoebae. For example, the effects of the age of the initial culture have become more obvious. In general, the amoebae from 4-day-old cultures, produce the flagellate form quickly and then revert quickly to the amoeboid form. Such amoebae are mostly large and active and very few are present as cysts. Five-day-old cultures have slightly smaller cells which react rather more slowly and are less regular in size. Still older cultures react even more slowly and irregularly, and more and more of the amoebae are found in the cyst condition.

There is still much that is not understood about the conditions of culture and their effects on the subsequent behaviour of the amoebae, for the amoebae from different stock cultures, in spite of rigid experimental conditions, are somewhat

variable in the way in which they respond to changes in their environment. Different preparations of amoebae, which appear to be closely similar, often give different results when subjected to apparently the same set of experimental conditions. This variability has always to be borne in mind in any interpretation of the results described. Some of the variability must arise from the manner in which the amoebae are grown on beef-extract-agar slopes with a somewhat unknown bacterial flora, and their state of nutrition may well influence the way in which the cells respond to the methods used for freeing them from bacteria for experimental purposes.

Two methods have been used for washing the amoebae and sometimes a combination of both was necessary. The amoebae have generally been washed by repeated centrifugation from relatively large volumes of buffer solution (B_2) but occasionally by allowing the cells to settle out from suspensions in B_2 on to the bottom of a clean and sterile Petrie dish about $1\frac{1}{2}$ in. in diameter. After about 20 min. in the dish, by which time the majority of the cells were actively creeping on the glass surface, the cells were very gently washed with frequent changes of B_2 till most of the bacteria were removed. This latter method is convenient when the bacteria from the stock culture tubes happen to be easily thrown down with the amoebae in the centrifuge. It is very satisfactory for obtaining clean, active, and more or less uniform amoebae, though the wastage is somewhat greater than by the centrifuge method. When the amoebae are thoroughly cleaned they can be readily dislodged from the glass by more vigorous washing and then concentrated by gentle centrifugation.

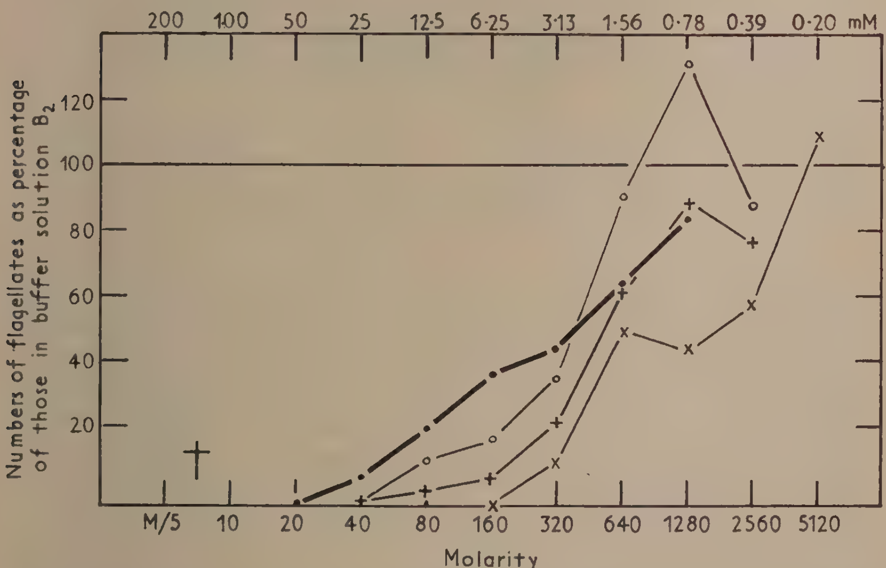
The effects of these 'washing' procedures on the amoebae may also contribute to the variability of behaviour. Many metazoan cells lose ribosenucleoproteins, and phosphates when washed in simple salt solutions, and there may be many other properties of the amoebae which change when the organisms are washed free from their normal growth medium and their usual bacterial population. Moreover, the importance of these losses or changes may well vary with the initial state of nutrition of the amoebae. Clearly, there would be enormous advantages in being able to keep *Naegleria* in a standard synthetic medium, and using the cultures at standard ages.

The pH of all solutions has been adjusted to pH 6·8, and within the duration of the experiments there has not been found to be any significant departure from this value. Appropriate adjustments have been made in all control solutions for alterations in the concentrations of other ions, caused by correction of pH.

EFFECTS OF CHOLINE AND RELATED COMPOUNDS

The investigations of Koch (1954) on the active uptake of sodium by the gills of the crab, together with the association of cholinesterase and acetyl choline with ciliated membranes (Bülbring, Burn, & Shelley, 1953; Kordik, Bülbring, & Burn, 1952) and in various ciliate (Bayer & Wense, 1936) or flagellate organisms

(Bülbring, Lourie, & Pardoe, 1949) suggested that choline and similar compounds might be of importance in relation to the ionic balance of these amoebae, particularly of those in the flagellate state. It is interesting, for example, that a trypanosome produces acetyl choline, but the more amoeboid malarial parasite does not (Bülbring, Lourie, & Pardoe, 1949). Moreover, the observations of Thomas (1936) and of Chèvremont & Chèvremont-Comhaire (1945) on the action of choline and related compounds in favouring the amoeboid (macrophage) form of cells in tissue cultures suggested that choline and quaternary ammonium compounds might favour the amoeboid form of *Naegleria*.



TEXT-FIG. 2. The action of choline and related compounds on the numbers of amoebae which became flagellate as a percentage of the number becoming flagellate in buffer solution (B_2) [=100]. Cross indicates death point for amoebae.

. ——— . NaCl (in water)   o ——— o Tetraethylammonium
+ ——— + Choline chloride   × ——— × Tetramethylammonium

For these and similar reasons amoebae were treated with serial dilutions of choline chloride after washing with several changes of distilled water or buffer-solution (B_2). The results are shown in Text-fig. 2 and it is evident that choline chloride acts on the amoeba in much the same way as does NaCl. It is not immediately toxic to the organism in the amoeboid form till a concentration of about 200 mM. is reached, but the flagellate form begins to be suppressed at concentrations higher than 0·5 mM. and flagellates are practically absent above about 25 mM.

When flagellates were placed in solutions of choline chloride (20 mM. and 6 mM.) there was an almost immediate reduction in their numbers as compared with controls simultaneously placed in distilled water (Table 2).

TABLE 2

Numbers of flagellates, which survive as such after treatment with quaternary ammonium compounds, as percentage of those which survive in the control medium

Compound	*Test solution* (mM/l.)	*Control medium*	*Duration of test* (hours)	*Percentage*
Choline	6·25	Distilled water	2	37·5
	20	Distilled water	5	17·5
Tetramethylammonium	10	B_2	5½	6
Tetraethylammonium	6·25	B_2	4½	46
	20	B_2	5½	12
Pentamethonium	10	B_2	¾	21
	10	B_2	8½	12
Hexamethonium	10	B_2	½	50
	10	B_2	8½	25

This reduction, without complete suppression, is somewhat difficult to interpret. It may indicate that the flagellate form is altered back to the amoeboid form by the choline, but that not all the cells are affected by the concentrations used, or it may be that there is normally some cycling between the amoeboid and the flagellate states by cells approaching equilibrium, and that choline prevents those cells which revert to the amoeboid form from again becoming flagellate.

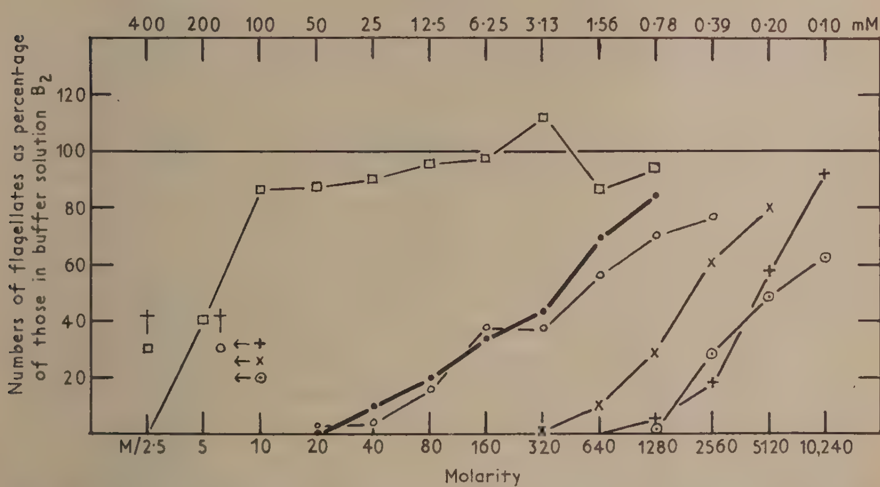
These results with choline suggested that perhaps acetyl choline would be more active than choline itself. However, this was not found to be so. Acetyl choline caused the amoebae to round up and become inactive at concentrations of about 0·5 mM. and higher, but in the experiments here described there was no indication of any differential action on the two forms at lower concentrations. Moreover, 1/3,000 acetyl choline added to a suspension of flagellates did not have any apparent and immediate effect.

Nevertheless, in view of Koch's observations on crabs' gills, the action of anticholinesterases was also investigated. The action of eserine sulphate (10^{-3}–10^{-6} M) was first tried. The results were anomalous. In three experiments fairly high concentrations of eserine (1 : 4,000 to 1 : 32,000) produced more than twice as many flagellates as the controls, but later repetitions (4 experiments) failed to show any such action. Concentrations of 1 : 1,000 and higher were toxic. In any case eserine never had any action at those concentrations in which, in other situations, it is known to poison cholinesterase (i.e. about 10^{-5} M), so that whatever may have been the cause of the anomalous results at concentrations around 10^{-4} M it was probably concerned with factors other than cholinesterase, and possibly with other esterases.

Similar results were obtained with DFP (diisopropylfluorophosphonate, kindly supplied by Dr. B. C. Saunders) for this inhibitor of cholinesterase showed little selective action on either the amoeboid or flagellate form. It became toxic to both at concentrations greater than about 10^{-4} M and flagellates occurred in normal numbers until concentrations greater than 10^{-5} M were used.

It may be concluded, therefore, that under the conditions of these experiments acetyl choline and cholinesterase are not immediately concerned with the change of form in the amoebae.

It appeared from these results that the action of choline was not augmented by its acetylation, and other substances related to choline were therefore examined.



TEXT-FIG. 3. Action of methonium compounds and other substances on the numbers of amoebae which became flagellate as percentage of the number becoming flagellate in buffer solution (B_2).

·——· NaCl (in water) o——o NH_4Cl □——□ Urea

×——× Decamethonium +——+ Hexamethonium ⊙——⊙ Pentamethonium

Crosses with appropriate symbols indicate death points for amoebae. Those with horizontal arrows indicate that the amoebae were still partially active at these concentrations which were the highest concentrations tested.

These included betaine, ammonium chloride, trimethylamine oxide, tetramethyl ammonium iodide and bromide, tetraethyl ammonium bromide and iodide, tetrabutylammonium chloride, pentamethonium iodide, hexamethonium iodide, and decamethonium iodide, as well as one or two other substances, e.g. cetyl trimethyl ammonium bromide containing quaternary ammonium groups. Ammonium chloride was found to behave very similarly to sodium chloride and to choline (Text-fig. 3). So also did the tetramethyl, tetraethyl, and tetrabutyl ammonium compounds. Trimethylamine oxide and betaine were both relatively

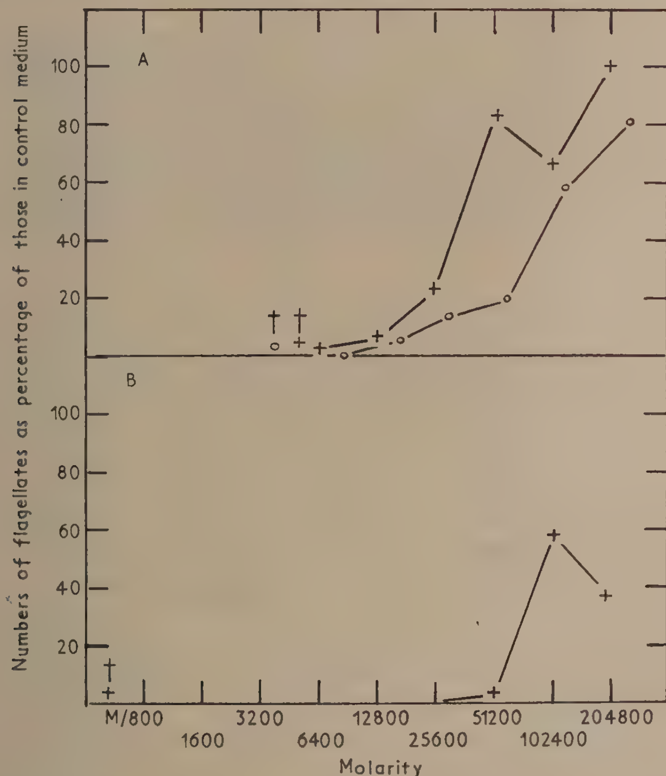
inactive. Cetyl trimethyl ammonium bromide was rather toxic even at 0·001 mM., but at great dilutions it showed some selective action in favour of the amoeboid form. The methonium compounds containing two quaternary nitrogen groups were very potent in suppressing the flagellate form and maintained the cells as amoebae over a very wide range of concentrations (100 mM.–1 mM.; Text-fig. 3). Like choline, these compounds when applied directly to the cells in the flagellate form also reduced the numbers of flagellates, but, as before, it was not clear whether this was a direct action or was due to reverting amoebae being fixed in the amoeboid form (Table 2).

All the quaternary ammonium compounds which suppressed the flagellate form also produced interesting morphological changes in the amoebae. In concentrations between 25 mM. and 3 mM. there was a great tendency for the amoebae to contain large vesicles, which often resulted in the cells becoming spherical and inactive. This inactivity, however, was not due to the toxicity of the compounds for at higher concentrations of the drugs the organisms again became actively amoeboid, though they then showed a sharp distinction between a granular endoplasm and wide hyaline ectoplasmic pseudopodia. This phenomenon was particularly noticeable with the methonium compounds, and is perhaps connected with a change in some of the cell proteins to a more coiled or contracted form (Marsland, 1956). Perhaps relevant to this are some unpublished observations by Mr. T. Vickers on the effects of decamethonium iodide and tetraethylammonium chloride on certain cells in both fish and rats. He found that these compounds produced well-developed intracellular canals in the exocrine cells of the pancreas and in the cells of the liver and kidney tubules, and that the oxyntic cells of the gastric mucosa showed large vesicles. A similar development of large vesicles occurs on treatment of the amoeba *Chaos chaos*, with 5 mM. adenosine triphosphate (Kriszat, 1950), on feeding *Paramecium* with thyroid (Shumway, 1917) and on treating *Paramecium* with adrenalin or with anterior pituitary extract (Flather, 1919).

The quaternary ammonium compounds were applied to the amoebae either as chlorides, bromides, or iodides. It was therefore necessary to investigate the actions of bromides and iodides to ascertain that the observed actions were not due to these anions rather than the cations. Bromides of sodium and potassium were found to behave very like the chlorides. The action of the iodides was, however, interesting in that not only could the iodide ion not be held responsible for the complete inhibition of the flagellate form, e.g. at 3 mM., as occurs with the methonium compounds, but in some experiments it actually caused an acceleration of the change to the flagellate form, particularly at concentrations more dilute than 1·6 mM. These results, however, like those with eserine were by no means uniform and there is still more to investigate in connexion with both these substances. It is perhaps of interest, however, to note that iodide is one of the substances which under some conditions promote 'animalisation' of developing embryos and its action on amoebae in favouring the flagellate form

may thus be similar and is to be contrasted with that of the 'vegetalizing' agent lithium which suppresses the flagellate form of amoebae.

Koch (1954) in his work on sodium transport in crabs' gill tissue observed that certain basic dyes, some of which contain quaternary ammonium groups act like



TEXT-FIG. 4. The actions of methylene blue and neutral red at different concentrations (abscissae) on the numbers of amoebae becoming flagellate as percentage of those becoming flagellate in control solution (ordinates).

A in Bicarbonate buffer solution

B in Distilled water

Crosses indicate death points.

+——+ Methylene blue o——o Neutral red

the simpler quaternary ammonium compounds and prevent the uptake of sodium into the blood. Among these dyes were methylene blue, brilliant cresyl blue, and neutral red. All three of these have been found to be strongly inhibitory to the change from the amoeboid to the flagellate form when applied in distilled water to *Naegleria gruberi*. Under these conditions methylene blue (Gurr's vital)

did not stain either the amoebae or the flagellates, apart from a few cytoplasmic granules which stained intensely, till applied in concentrations greater than 1·2 mM., at which point the amoeba became round and inactive. On the other hand, the cysts became very quickly and intensely stained in even the most dilute solutions. The flagellate form was rarely seen in concentrations of methylene blue higher than 0·02 mM. (Text-fig. 4).

The physiological actions of methylene blue are certainly various, but it is of interest that this action in suppressing the flagellate form of the amoeba could well be similar to the action on sodium transport in the gills of the crab. In the gills the sodium uptake is inhibited by the presence of methylene blue on the outside of the gill membrane. When applied to *Naegleria* in the flagellate phase it causes an immediate change to the amoeboid form, i.e. it suppresses the form in which sodium accumulation is proceeding. Perhaps it actually blocks the sodium mechanism.

There is, however, another side to the action of methylene blue on the amoeba. When it was applied, not in water, but in a buffer solution containing $NaHCO_3$ (see Willmer, 1956) it became toxic to the amoeboid form in much lower concentrations, so that it had very little differential action in suppressing the flagellate form. It will be remembered (Willmer, 1956) that the action of the bicarbonate ion was also important in determining the behaviour of the amoeba in response to the presence of lithium salts and to sodium salts other than the bicarbonate. Possibly these differences may be caused by the penetration of the cell by CO_2 from the bicarbonate solution and the consequent change to acidity on the part of the cell contents, though it is not immediately clear why this should increase the toxicity of methylene blue to the amoeboid form and somewhat decrease its effectiveness on the flagellate form. It could be suggested that the methylene blue might be more ionized in the acid cell contents and that its toxic effects are dependent on its action as a cation.

As already mentioned, neutral red behaves in a manner somewhat similar to methylene blue and brilliant cresyl blue. It accumulates to some extent in a few cytoplasmic granules, rather more when the cell is in the amoeboid form than in the flagellate form but it is not as actively accumulated as it is for example by some other living cells, e.g. macrophages in tissue cultures of vertebrate tissues. It often accumulates strongly in the uroid region of those cells which have developed a definite polarity, in a manner similar to that described by Goldacre (1952) for the limax form of *Chaos chaos*.

ON THE METABOLISM OF THE AMOEBAE IN RELATION TO THEIR FORM

If, as the hypothesis outlined above suggests, the change of form of the amoeba is related to the accumulation or excretion of cations, then at all concentrations except the equilibrium concentration the cell must either become relatively impermeable or must do work to maintain its ionic balance. Sub-

stances which affect biological membrane permeability and those which alter metabolic rate are therefore both likely to have effects on the 'metaplasia' of amoebae. For example, an amoeba placed in distilled water presumably tends to lose ions and it changes to the flagellate form to counteract this. Anything which decreases permeability must delay this change by decreasing the loss. The action of Mg^{++} may be a case in point, for this ion definitely delays the change and in other situations, e.g. certain nerve-cells (del Castillo & Engbaek, 1954) where it is known to prevent the release of acetyl choline, it certainly causes a decrease in permeability. It may, of course, have other actions also. In bacteria, for instance, it affects the synthesis of amino acids which in itself may have far-reaching effects on the ionic content of the cell. In amoebae the addition of 3·1 mM. $MgCl_2$ to cells in the flagellate form virtually stops their swimming within half an hour. The effect, however, is not immediate as in the case of methylene blue.

TABLE 3

Numbers of flagellates, as such, after 6 hours approximately in test solutions as percentage of those in water at the same time

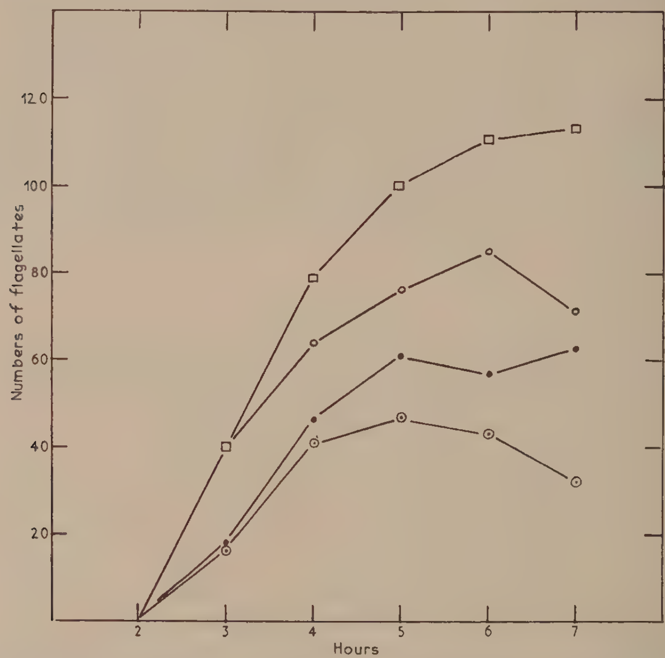
Test solution	*Flagellates*
3·1 mM. $MgCl_2$	3
3·1 mM. $CaCl_2$	22
3·1 mM. $MgCl_2$ + 3·1 mM. $CaCl_2$	30
3·1 mM. KCl	54
3·1 mM. KCl + 3·1 mM. $MgCl_2$	12

The action of Mg^{++} on nerve-cells can be reversed by the simultaneous presence of Ca^{++} (del Castillo & Engbaek, 1954) and this has also been found to be true for its action on amoebae (Table 3).

Quaternary ammonium compounds have also been shown to prevent the liberation of acetyl choline from nerve cells (Paton & Perry, 1953), so these substances also may be acting on amoebae by virtue of some similar action on permeability. On the other hand, there are many other ways in which both these groups of substances could be involved, and apart from indicating a certain similarity between amoebae and some nerve-cells in the manner in which they each respond, the observations by themselves throw little light on how the ionic regulation of the amoeba is carried out.

When amoebae were placed in buffer solution (B_2) the flagellate form began to appear in increasing numbers after about 2 hours, and then, after about 6 or 7 hours their numbers again began to fall off (Text-fig. 5). With concentrations of NaCl up to 2 mM. added to the buffer at the beginning of the experiment there was little difference to be noted in the response of the amoebae until after about 4 hours, nearly as many flagellates being formed as in the buffer solution

alone. From that time onwards, however, the number of flagellates became definitely less than in the buffer solution. This result would follow if the flagellate cell were able to concentrate the sodium ions within itself, or if the sodium ions after penetration helped the cell to pick up potassium ions from the buffer solu-



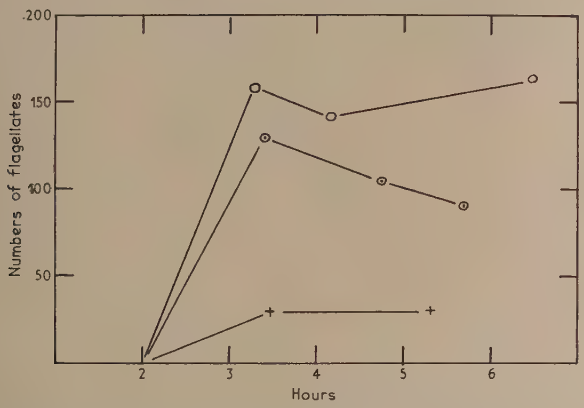
TEXT-FIG. 5. Summary of experiments showing the effect of glucose and sodium chloride on the numbers of cells in the flagellate form during the first 7 hours after the addition of these substances, in buffer solution (B_2) to freshly washed amoebae. All figures have been expressed as a percentage of the numbers of flagellates in the buffer solution after 5 hours.

□———□	Buffer solution (B_2)		
○———○	„	„	+6·7 mM. glucose
•———•	„	„	+6·7 mM. NaCl
⊙———⊙	„	„	+6·7 mM. glucose+6·7 mM. NaCl

Note the more immediate action of the NaCl and the delayed action of the glucose.

tion, which contains 0·2 mM. KCl, as they do in other similar situations. The former is the simpler hypothesis and as yet there is little to suggest that this amoeba discriminates, except quantitatively, between these cations. It may be that it normally accumulates whichever monovalent cation is available, or, if more than one is available, the proportions of each which enter may be partly determined by ionic size or mobility in the membrane.

At concentrations of NaCl higher than 6·7 mM. the numbers of amoebae which initially turned flagellate became noticeably reduced (Text-fig. 6). At this stage the loss of cations to the external medium presumably becomes insignificant and the necessity to 'switch on' the concentrating mechanism is reduced. This would be consistent with a total cation content of about 8 mM. which is approximately that found by Carter (1957) for the ciliate but is probably



TEXT-FIG. 6. An experiment showing the effect of different concentrations of sodium chloride in the presence of glucose.

o ——— o Buffer solution (B_2) + 6·7 mM. glucose
⊙ ——— ⊙ " " + 6·7 mM. glucose + 2 mM. NaCl
+ ——— + " " + 6·7 mM. glucose + 20 mM. NaCl

rather lower than that in *Naegleria* if the preliminary estimates are confirmed.

If instead of NaCl, glucose (final concentration 6·7 mM.) was added to the buffer solution at the beginning of the experiment an exactly similar result followed (Text-fig. 5). There was no initial difference in the numbers which turned flagellate, but after about 4 hours the tubes containing glucose showed progressively fewer and fewer swimming forms. When glucose was added in distilled water it had no apparent effect on the numbers of cells which were flagellate after 6 hours (Willmer, 1956) so it is unlikely that these effects of glucose are purely osmotic.

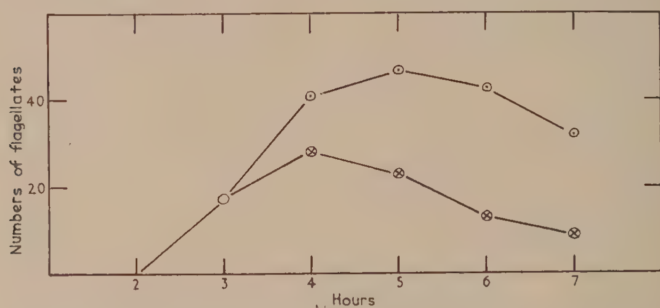
Thirdly, the simultaneous addition of NaCl (final concentration 2 mM. to 6·7 mM.) and glucose (final concentration 6·7 mM. to 10 mM.) to the buffer solution enhanced the delayed effect still further.

These results, which are illustrated in Text-figs. 5 and 6, strongly suggest that the flagellate form of the cell has an active mechanism for capturing positive ions and this is assisted by further supplies of glucose. While the possibility remains that the action of the glucose is concerned with preventing the loss of ions, rather than with actively assisting in their accumulation, the time-scale of

events points to the latter, and it may therefore be tentatively concluded that the flagellate form has an inwardly directed cation pump.

Certain similarities with nerve-cells have already been mentioned and some nerve-cells are known (Krebs & Eggleston, 1949; Weil-Malherbe, 1950) to have a mechanism dependent on glutamic acid and glucose for the accumulation of their intracellular potassium. Thus the possibility of a 'glutamate mechanism' for maintaining the cation content of *Naegleria* seemed to be worth investigating.

When sodium *l*-glutamate was added to a glucose-containing medium it was



TEXT-FIG. 7. Summary of experiments comparing the effects of sodium chloride and sodium *l*-glutamate on the numbers of cells in the flagellate form in the first 7 hours after the addition of these substances to freshly washed amoebae in buffer solution (B_2). All figures as percentages of numbers of flagellates at 5 hours in buffer solution (B_2).

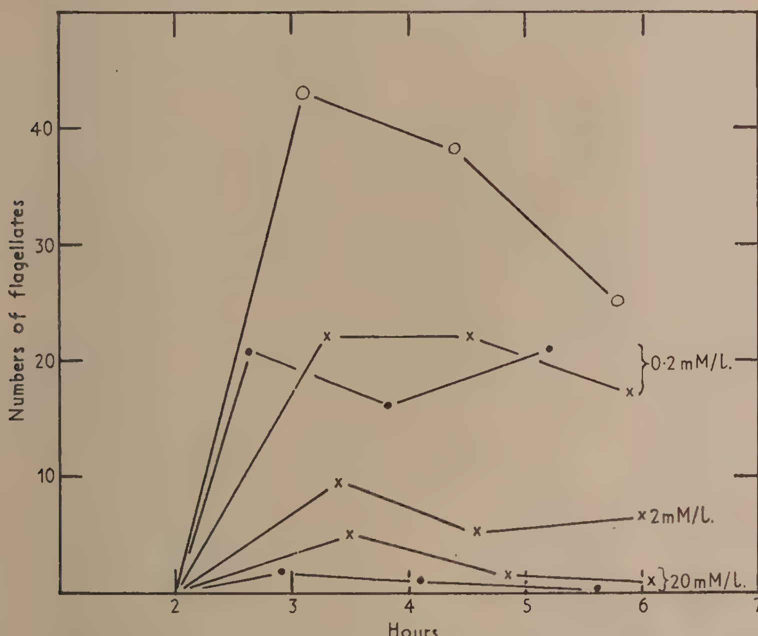
⊙——⊙ Buffer solution (B_2)+6·7 mM. glucose+6·7 mM. NaCl

⊗——⊗ „ „ +6·7 mM. glucose+6·7 mM. Na *l*-glutamate

found to have a much greater action than the corresponding concentration of NaCl in hastening the return of the flagellates to the amoeboid form (Text-fig. 7). Thus the similarity with nerve-cells appeared to be capable of further extension. However, the action of the glutamate was not found to be so specific for amoebae as it apparently is for nerve-cells, because it can be replaced very effectively with sodium α-ketoglutarate (Text-fig. 8), which is not so for nerve-cells.

The addition of glutamine to the amoebae in buffer solution generally produced similar results to those produced by sodium glutamate and these were dependent on the simultaneous presence of glucose (Text-fig. 9). The action of glutamine was, however, not so regular or predictable as that of sodium glutamate and, indeed, in certain experiments it appeared to have no action at all. Possibly it has to be converted to glutamic acid before it is effective and, in support of this, there was some indication that freshly made solutions were less effective than those which had been stored at about 3° C. for 24 hours and in which bacteria could have caused some conversion. Alternatively the amoebae may sometimes be so well nourished as to make additions of this sort from the

outside quite ineffective. There was, in fact, a general correlation between the age of the culture used and the magnitude of the effect produced by glutamine. The very well-fed cells of the 4-day culture were less influenced by added glutamine and glucose than those from 6- or 7-day cultures.



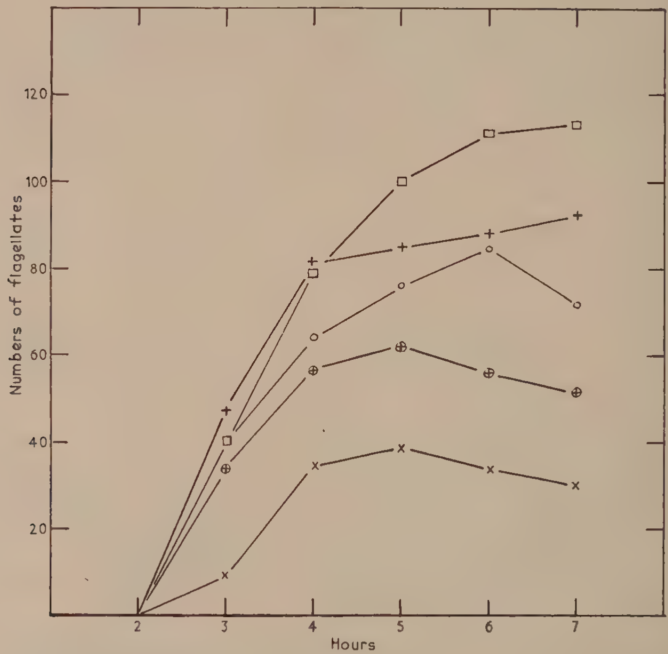
TEXT-FIG. 8. An experiment comparing the actions of sodium *l*-glutamate and sodium α-ketoglutarate on the numbers of cells becoming flagellate.

o——o Buffer solution (B_2) + 6·7 mM. glucose
×——× „ „ + sodium *l*-glutamate
•——• „ „ + sodium α-ketoglutarate

Asparagine was found to behave like glutamine so that again there is probably very little specific about these compounds in relation to the metabolism required for the 'cation pump'. Nevertheless, the fact that these compounds affect the working of the pump makes it fairly clear that an active process is involved in causing the cells to revert to the amoeboid form.

As already pointed out, these substances were not so effective immediately as they became after the lapse of some hours; that is to say they did not lengthen the lag period before flagellates appeared nor, in the lower effective concentrations at least, did they at first greatly alter the rate at which the amoebae became flagellate. This was particularly true for the mixtures of glutamine and glucose (Text-fig. 9). In the case of sodium glutamate the position has to be controlled with reference to the effects of the added sodium. When, however, allowance

was made for this by comparison with additions of sodium chloride (6·7 mM.) then the glutamate at the same concentrations was seen to have very little immediate effect on the transformation to the flagellate form (Text-fig. 7), and the lag period was not lengthened any more than by the sodium added.



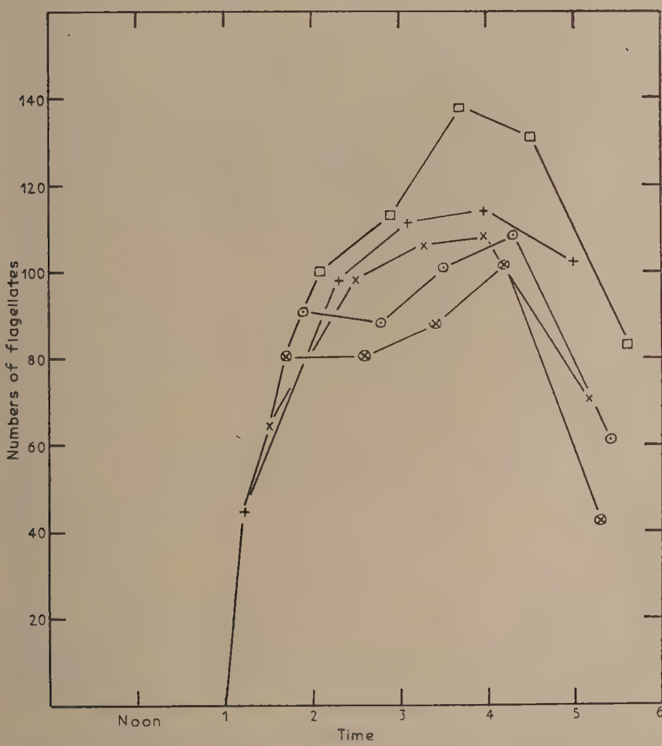
TEXT-FIG. 9. Summary of experiments showing the effect of glutamine on the numbers of cells which become flagellate in the first 7 hours after its addition to amoebae in various media. All figures are percentages of the number of flagellates after 5 hours in buffer solution (B_2).

□——□	Buffer solution (B_2)		
+——+	,,	,,	+6·7 mM. glutamine
o——o	,,	,,	+6·7 mM. glucose
⊕——⊕	,,	,,	+6·7 mM. glucose+6·7 mM. glutamine
×——×	,,	,,	+6·7 mM. glucose+6·7 mM. NaCl

Note that only in the medium containing NaCl is there any significant immediate effect.

In contrast to its lack of action on the amoeboid form, sodium glutamate when added to the amoebae after they had already become flagellate had an immediate action in reversing the change (Text-figs. 10–13). There are certain technical difficulties about preparing large numbers of cells in the flagellate form for inoculating into series of experimental tubes in a manner which allows comparable results to be obtained, so in these experiments the amoebae (as such)

were inoculated into the experimental tubes each containing 0·5 c.c. of B_2 only; then, after about 4 hours, when the number of flagellates was still increasing but nearly at the maximum, another 0·5 c.c. of experimental solution of twice the required strength was added. Counts of amoebae were made immediately before



TEXT-FIG. 10. An experiment comparing the effects of constituents of the 'glutamic system' on amoebae already in the flagellate form.

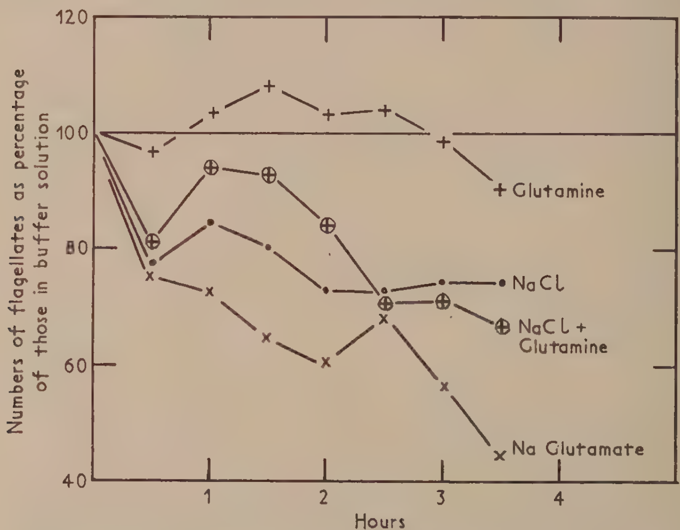
□———□ Buffer solution (B_2)
+———+ 2 mM. glutamine
×———× 2 mM. glutamine+2 mM. NaCl
⊙———⊙ 2 mM. NaCl
⊗———⊗ 2 mM. Na *l*-glutamate

The reagents were added immediately before the first points marked with the appropriate symbols.

the addition and as often as possible afterwards. The method is not entirely satisfactory since, because of the time consumed in counting them, the amoebae cannot always be caught at the same state of the tide of change to the flagellate form and this makes the data somewhat difficult to standardize. For example, a tube caught early in the process towards flagellum formation may actually

show more flagellate cells after the addition of an inhibitor than before, while another control tube taken after the turn of the tide may show fewer flagellate cells than before the addition of the control B_2 solution. Controls therefore have to be used continually throughout the experiment. While this method is clearly not ideal, the accompanying figures show that definite results can be obtained by its use.

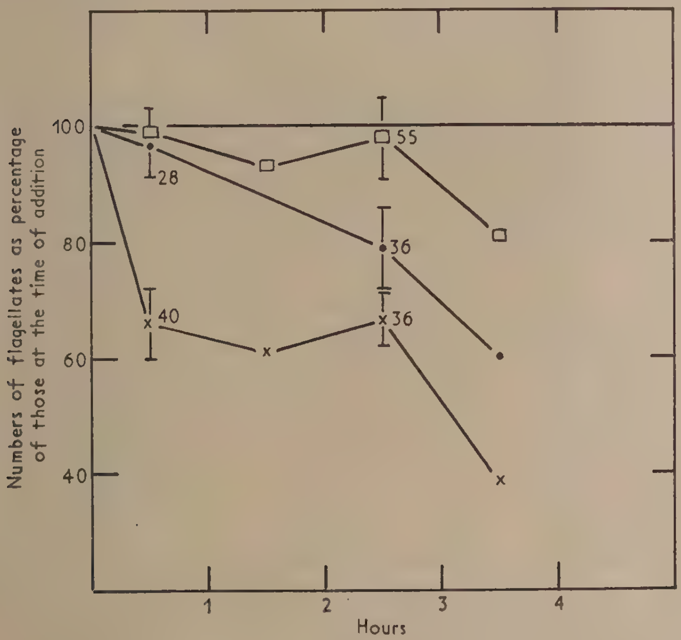
Text-fig. 10 shows the results of a particular experiment in which the actions of glutamine, sodium glutamate, and sodium chloride have been compared. Glucose was present in all cases. It will be seen that after the addition of glutamine the numbers of flagellates at first followed a course very similar to that of



TEXT-FIG. 11. The combined results of two experiments of the type shown in Text-fig. 10 showing the actions of glutamine (+), NaCl (.), Na *l*-glutamate (x), and a combination of NaCl and glutamine (⊕) in reducing the numbers of flagellates when added in 2 mM. concentration to cultures in which flagellates were numerous.

the controls in buffer solution alone. Only later did the curve diverge from the B_2 curve and fall appreciably below it. On the other hand, with both sodium chloride and, particularly, sodium glutamate the number of flagellates was immediately reduced. Repetition of similar experiments led to the results depicted in Text-fig. 11, and it is evident that sodium glutamate had an immediate and direct action but glutamine had none or only a very delayed one either in the presence or absence of NaCl. This again can be taken as an argument that glutamine only becomes effective after some hydrolysis, or that it cannot act immediately on the flagellate form but only after penetrating the cell in the amoeboid form.

In these experiments the sodium glutamate reduced the numbers of flagellates almost immediately and continued to do so, while the chloride, though sometimes causing an initial drop, acted in general much more slowly. Text-figs. 12 and 13 illustrate the results obtained from a long series of experiments in which NaCl and Na *l*-glutamate were added to amoebae in the flagellate form and the



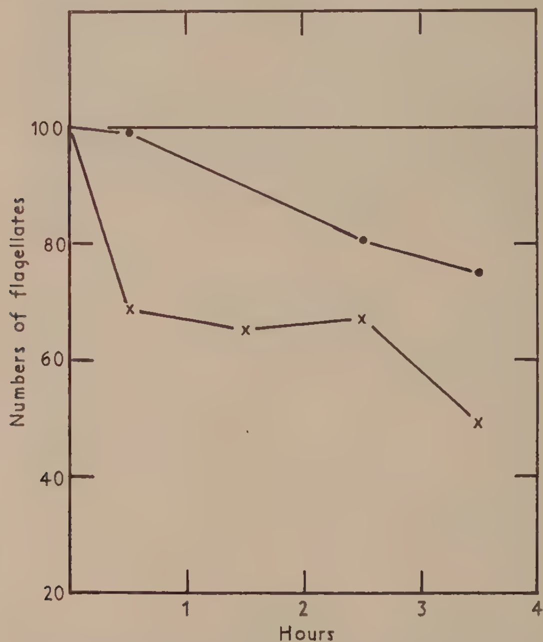
TEXT-FIG. 12. Summary of all experiments in which 2 mM. NaCl and 2 mM. Na *l*-glutamate have been added to amoebae in the flagellate form. All figures expressed as percentage of the number of flagellates at the time of addition of experimental medium.

□ Buffer solution . NaCl x Na *l*-glutamate

Vertical lines represent twice the standard error of the mean and the figures the numbers of readings. Each point represents the mean for all readings within the hour.

different timing of the effects of the two substances is clearly seen. This difference may simply be the result of different rates of penetration but it is exactly what would be expected if the entry of sodium was normally proceeding slowly in accordance with its availability in the external medium, and if the uptake could be further accelerated by a metabolic process involving in this case the use of glutamic acid and glucose. These results were all obtained with 2 mM. concentrations of chloride and glutamate and if the concentrations were increased to 10 mM. the difference between the chloride and the glutamate practically disappeared. Presumably the ionic balance of the cells no longer required the

active collection of sodium ions from the external medium at that concentration. The conclusion seems inevitable therefore that in the flagellate form *Naegleria gruberi* has a metabolic mechanism, involving glucose and glutamic acid or other similar constituents of the metabolic pool, for the accumulation of cations



TEXT-FIG. 13. The same figures as in Text-fig. 12 but plotted as a percentage of the figures for the buffer solution at each point.

. NaCl x Na *l*-glutamate

from the medium. In general, there seems to be little discrimination between Na^+ and K^+. A pure K-phosphate buffer was found to give results which did not differ significantly from those obtained either in a pure Na-phosphate buffer or in a mixed Na-K-phosphate buffer. In one or two experiments in which both Na^+ and K^+ were present the amoebae became flagellate somewhat earlier than in solutions with either cation alone, and they returned more quickly to the flagellate form. The results, however, were not statistically significant.

DISCUSSION

As emphasized in a previous paper, *Naegleria gruberi* is an interesting organism for the study of differentiation in that it changes its morphology under the influence of changed external conditions, and consequently can be used as a test

object for investigating the nature of the processes which have to take place during the change of form.

It now seems to be certain that a primary cause of the 'metaplasia' from amoeba to flagellate is a drop in the cation content of the surrounding medium and the change can be inhibited by raising the ionic content of the medium.

Naturally not all cations are equally effective; Li^+ and Mg^{++} ions, for example, tend to inhibit the change to the flagellate form in very low concentrations. Na^+, K^+, and Ca^{++} on the other hand, while showing some individual differences act over rather similar concentration ranges but at a higher level than Mg^{++}. Probably there are definite interactions between these ions, as there are for nearly all other organisms, but only the reduction of the Mg^{++} effect by Ca^{++} has so far been studied. Furthermore, it has been shown that the presence of certain anions in the environment, e.g. bicarbonate, phosphate, and lactate, may favour the change towards the flagellate form, while others, e.g. sulphate, have exactly the opposite action.

The actions of these various ions may of course, be many and various, e.g. on surface charges, on membrane permeability, on colloidal state, on internal pH, on chemical composition, on enzymic activity, and on the orientation of protein particles, to mention only a few. It is unwise therefore to expect, at this stage, to be able to sort out exactly what is happening in the amoeba as the external ionic content is being changed.

Nevertheless, if a cell contains a certain amount of protein, and other complex ions will of course enter into this category also, then the state of that protein must depend on the balance of positive and negative ions in its immediate surroundings, because these affect the numbers of charged groups on the proteins themselves and no doubt the state of the protein, i.e. as a coiled or extended chain, may affect the numbers of groups which could bind other ions. It is easy to imagine, therefore, that any particular cell, in order to carry out its normal functions should have a definite requirement for its internal ionic content. In the case of *Spirostomum* this appears to centre round K^+ at 7 mM./l. and Na^+ at 1 mM./l. If the cell were to synthesize more protein or accumulate amino acids these figures might have to be raised; conversely a forced change in ionic content could well lead to alteration in the amount, distribution, or state of aggregation of the cell proteins. A cell, then, in order to preserve its *status quo*, should be expected to have some sort of ion-regulating mechanism, and, conversely, changed ionic content of the medium, if it causes changed internal content also, should be expected to cause changed morphology. In *Naegleria gruberi* these correlations seem to be borne out, and the amoeboid form with its lobose pseudopodia, random shape, relative lack of orientation, roving contractile vacuole, highly granular cytoplasm with hyaline ectoplasmic processes is characteristic of the state in which the external cation concentration probably exceeds the internal. In this state one would expect water to be drawn out of the cell, and the contractile vacuole to be unnecessary. However, the vacuole

continues to function till much higher concentrations are reached, and it seems possible that an active engulfing of water may have to take place to prevent dehydration, and that excess ions simultaneously imbibed are then eliminated through the contractile vacuole. Something of this sort may lie at the root of the somewhat mysterious process of pinocytosis, which could then be seen as a mechanism by which a cell could retain an ionic balance in a situation in which the external medium is more concentrated than that required for the equilibrium of the cell.

The flagellate form of *Naegleria*, with its definite orientation and polarity, its contractile vacuole in a fixed position, its more rigid surface and its inability to form pseudopodia, is on the other hand characteristic of the conditions imposed by an external cation concentration lower than the internal. The flagellate cell may perhaps have its proteins in a more fibrous form, as suggested by the ability to form flagella and filamentous rather than lobose pseudopodia. These fibrous proteins would be expected to have more exposed polar groups capable of binding ions. When these proteins change to the globular form they drop these ions. It seems possible to believe then that in a given environment the amoeboid form would have a lower ionic content than the flagellate form. It would also have a mechanism for eliminating ions while the flagellate, requiring more ions, has a mechanism for their accumulation. These pumps could well be based on the fibrous-globular proteins themselves as has been suggested by Goldacre (1952).

Since the flagellate state can be brought about by the presence in the environment of anions which easily penetrate cells, e.g. HCO_3' which can penetrate as CO_2, lactate and low concentrations of phosphate, it is possible that an increase in internal hydrogen ion concentration may result and this then lead to the assumption of the flagellate form, while on the other hand increase in the internal OH^- concentration as by the penetration of NH_3 would by the same token favour the amoeboid form.

The experiments with glucose, glutamic acid, and sodium chloride indicate that ionic regulation is not a passive process in these amoebae and that the flagellate form actively absorbs cations from the surroundings. The fact that ketoglutaric acid is an effective substitute for glutamic indicates that there is nothing very specific about the glutamic acid as there is in the case of nerve-cells. In all these experiments, however, the amount of reserves available within the cell is an unknown quantity and the fact that ketoglutaric acid helps the pumping mechanism does not necessarily mean that glutamic acid is not involved. Indeed, the way in which glutamine behaves shows that glutamic acid is probably a key substance and its concentration in the cell may be maintained from either internal or external sources.

Into this picture the quaternary ammonium compounds could be fitted in several places and there is as yet little evidence as to which is most likely. They could alter the surface and prevent the loss of ions, and the formation of vesicles

in the higher concentrations perhaps suggests some interference with the normal excretion processes of the amoeboid form. Alternatively, if they enter the cell they presumably raise the cation content and so help to maintain it above the critical value. Since methylene blue penetrates the cell and acts in the same way as the other quaternary ammonium compounds this is at least a possibility, but against this it should be noted that the methylene blue is under these circumstances and everywhere except in a few granules reduced to the leucoform in which it is no longer ionic or a quaternary ammonium compound.

While the experiments described in this paper provide grounds for believing that the flagellate form of *Naegleria* has an inwardly-directed 'cation pump' which depends on active metabolic processes, there is nothing in them which indicates definitely that in the amoeboid form there is any comparable process acting in the opposite direction. It seems probable that there is, but experimental evidence is needed. A contractile vacuole is present in both states, and there may be more than one in the amoeboid form, but, so far, there is nothing known about the contents. Data on the ionic composition of *Naegleria* in its two states, comparable to those obtained by Carter (1957) on *Spirostomum*, would be invaluable.

Finally, it will be remembered that these experiments on *Naegleria* were initiated in order to explore the possibility that in this organism there was present at different times the same type of differentiation as is found at different places among the cells of the embryos of the most primitive metazoa, e.g. in the 'animal' and 'vegetal' poles of the various forms of amphiblastulae. A few comments on the results from this point of view may not therefore be out of place.

In an amphiblastula a cavity is formed within a single layer of cells, and, within limits, this cavity neither swells nor shrinks. When tissue cultures are made of portions of the mesonephros of chick embryos the pieces of nephric tubules tend to form little closed vesicles, and in the normal tissue culture medium those from the proximal tubules tend to swell up with increasing amounts of contained fluid, while those from the distal tubules, however, tend to collapse to solid masses of cells (Chambers & Kempton, 1933). This would happen if the proximal tubules tended to expel material or fluid into the cavity and the distal tubule cells tended to extract materials from the cavity. Since the amphiblastula neither shrinks nor swells, and if it is a closed vesicle like those formed by the nephric tubules, there are two possibilities. Either the cells contribute nothing and take away nothing from this cavity, which seems inherently incredible, or else they regulate its volume very accurately, and could well do this by opposing two mechanisms. It is worth considering then whether the flagellate anterior cells of the amphiblastula of the sponge contribute towards ionic stability by accumulating ions from the environment, while the posterior granular cells eliminate any excess ions which may arise. There is, of course, one very large difference between the state of affairs in the amoeba and that in

the amphiblastula. In the latter, the external surface of all the cells is exposed to one environment while the internal surface may meet another; in the amoeba the whole surface of the cell is exposed to one environment only. In the amphiblastula, therefore, the activities of both 'faces' of the epithelial cells have to be considered separately, but there is no reason to believe that this necessarily detracts from the idea that the amphiblastula is a stable structure because it combines cells, not only with different feeding mechanisms, and with different methods of locomotion, but also with differently orientated mechanisms for regulation of their ionic content and that of the fluids bathing their surfaces. If this is so, then it may be a principle which is capable of extension into other fields, e.g. to various epithelia which contain two types of cell and which are concerned with regulating the composition of the fluids on either side of them.

The recent observations of Tuft (1957) on the fluid accumulation in the cavities of the Xenopus embryo speak strongly in favour of ionic regulation and forcible movements of fluid. Indeed, it may be a useful concept in relation to embryonic development in general, that particular groups of cells can by their orientation of water- or ion-pumping mechanisms create local environments of specific ionic content for cells in their immediate neighbourhood. For example, cells lying below part of the ectoderm which is accumulating, say K^+, from the environment may well be forced to assume the form in which they prevent K^+ ions entering them excessively. The movement of water through cells may also produce a similar state of affairs and in such cases morphogenetic movements may actually be induced by the pressure changes involved.

SUMMARY

1. Further observations have been made on the effects of various substances on the change of form from amoeba to flagellate and vice versa in *Naegleria gruberi*.

2. Mg^{++} reduces the numbers of flagellates formed from amoeba and favours the conversion of flagellates to amoebae. Its action can be partly offset by the addition of Ca^{++}.

3. Methylene blue and other basic dyes suppress the change towards the flagellate form, particularly in distilled water, less so in a bicarbonate-buffer.

4. Substances containing a quaternary NH_3^+ generally suppress the change to the flagellate form. Methonium compounds are particularly effective. At certain concentrations these substances cause vesicles in the cytoplasm.

5. *l*-glutamine and Na *l*-glutamate applied to the amoebae reduce the numbers of flagellates which would otherwise form after a few hours in buffer solution.

6. Asparagine and Na α-ketoglutarate have similar effects.

7. Na *l*-glutamate (but not glutamine) applied to the flagellate form immediately reduces the numbers of flagellates. An equivalent amount of sodium chloride acts much more slowly.

8. It is suggested that the flagellate form of the amoeba has a mechanism for maintaining a stable internal cation concentration. This mechanism requires metabolic energy. Reduced permeability and more active expulsion of water are probably not adequate to explain the facts and an inwardly-directed cation pump is suggested for the flagellate form.

9. The application of ideas engendered by this work to other fields of cell differentiation are briefly considered; in particular, the concept of ionic regulation by the amphiblastula stage of invertebrates is discussed.

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Le développement du péroné dans les expériences sur la régulation des déficiences et des excédents dans la patte du Poulet

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SI le but des expériences sur la régulation est d'obtenir un développement correct et harmonieux d'un organe ou d'un organisme à partir d'une ébauche anormale, il n'est pas moins vrai que l'attention est souvent attirée par les individus s'écartant de façon plus ou moins importante du type parfait. Ceux-ci permettent souvent des conclusions intéressantes sur les mécanismes de la régulation.

Les résultats, des expériences relatées ici, ont été décrits antérieurement (Wolff & Hampé, 1954; Hampé, 1956, 1957).

SUPPRESSION DU PÉRONÉ AU PROFIT DU TIBIA

Dans une série d'expériences sur la régulation des déficiences intermédiaires (Wolff & Hampé, 1954) on découpe une tranche intermédiaire du bourgeon de membre au stade 19 à 20 de Hamburger & Hamilton (fig. 1*a*) et on remet la calotte en place sur le moignon (fig. 1a_1). La tranche intermédiaire excisée est greffée dans l'allantoïde d'un autre embryon (fig. 1b_1). On sait, en se référant à la topographie des ébauches présomptives et aux pièces osseuses du greffon développé, que la partie excisée comprend une partie des territoires distaux du futur fémur et des territoires proximaux des tibia et péroné. L'ensemble moignon plus calotte comprend alors les ébauches incomplètes du fémur, du tibia et du péroné.

Après l'opération les embryons continuent leur développement jusqu'au 10e jour où ils sont fixés, colorés et éclaircis. Chez deux embryons sur cinq, où la calotte est restée en place, la patte opérée est de longueur normale. Les différents segments sont bien proportionnés et les pièces osseuses bien formées. On constate toutefois l'absence de péroné.

Les pattes opérées des 3 autres embryons ont une constitution assez déficiente, certaines pièces osseuses sont manquantes ou fusionnées avec d'autres. A ces anomalies s'ajoute régulièrement l'absence de péroné.

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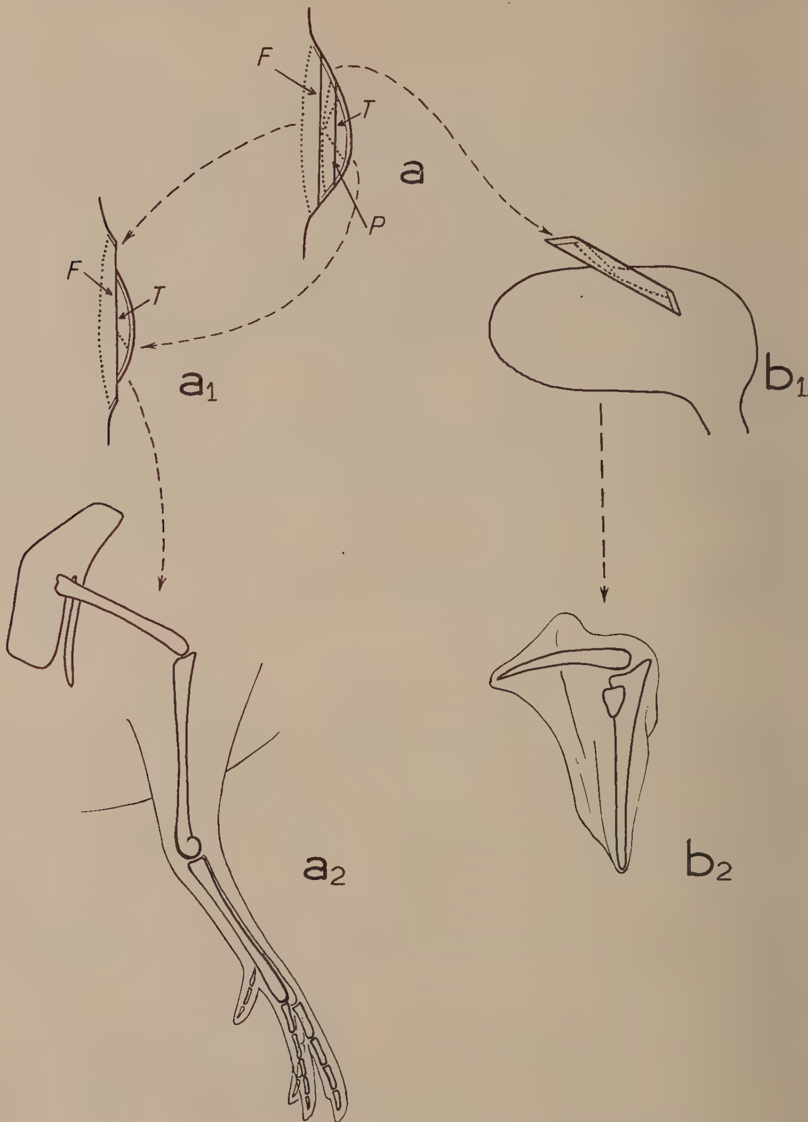

FIG. 1. Régulation des déficiences. Absence de péroné.

Fig. *a*. Le bourgeon de la patte au stade de l'opération. Le pointillé délimite les territoires pré-somptifs: fémur (*F*), tibia (*T*), péroné (*P*). En traits pleins, la zone excisée.

Fig. a_1. Le moignon coiffé de la calotte.

Fig. b_1. La partie médiane excisée et greffée dans l'allantoïde.

Fig. a_2. La patte développée ne présente pas de péroné.

Fig. b_2. Le greffon développé et composé du fémur, du tibia et du péroné.

L'étude détaillée du zeugopode de tous les embryons opérés à ce stade révèle l'absence régulière de péroné, par contre on est surpris de constater le développement correct du tibia. Or on sait que, dans l'opération précédente, on avait excisé du matériel présomptif aussi bien du tibia que du péroné. On aurait donc pu s'attendre à un développement réduit de chacune de ces deux pièces osseuses; or aucun des cas observés ne donne ce résultat. Le tibia se développe toujours en premier lieu, la reconstitution du tibia semble bénéficier d'une priorité. On n'a jamais observé un péroné bien développé à côté d'un tibia incomplet.

Il y a des cas où le tibia est incomplet et se termine en pointe, par exemple, lorsque la calotte terminale n'est pas restée en contact avec le moignon du bourgeon de patte; mais dans ce cas il n'y a pas trace d'un péroné.

Tout semble se passer de la manière suivante: lorsque le territoire présumé du zeugopode est lésé, tout le matériel restant de ce segment contribue à la formation du seul tibia; le péroné ne semble se constituer que lorsqu'il y a plus de matériel qu'il n'en faut pour l'édification du tibia.

Si par contre on opère à un stade plus avancé (21 à 22 de Hamburger & Hamilton), la partie excisée intéresse la région distale du tibia et le territoire proximal des tarsométatarsiens. Le zeugopode de la patte développée est alors normal, le tibia et le péroné sont normalement développés. D'après cela le territoire présomptif du péroné ne semble servir qu'à l'édification du tibia lorsque le territoire présomptif de celui-ci a été endommagé, par contre il ne semble pas contribuer à l'édification des tarsométatarsiens lésés.

FORMATION D'UN PÉRONÉ COMPLET

Pour obtenir la régulation des excédents (Hampé, 1956), on place deux bourgeons de membre l'un à la suite de l'autre, en enlevant la partie terminale d'un bourgeon au stade 22 (fig. 2*a*) et en remplaçant la partie enlevée par un bourgeon entier plus jeune (fig. 2*b*). Il y a donc une succession anormale des ébauches présomptives dans le complexe ainsi composé, puisque certaines ébauches sont représentées deux fois (fig. 2a_1).

Les résultats sont assez variables; dans le meilleur cas, la patte dérivant d'un tel complexe présente une conformation parfaitement normale.

Dans 7 cas cependant, la succession des éléments de la patte est normale et chez la plupart d'entre eux la longueur des segments l'est aussi, mais l'étude détaillée révèle une anomalie dans le développement du péroné: celui-ci est beaucoup plus long que normalement (fig. 2A).

Cette longueur inaccoutumée du péroné est due évidemment à l'excédent de matériel fémur, tibia et péroné. Il est surprenant de constater que le tibia ne dépasse pas sa taille habituelle mais que, par contre, c'est le péroné qui devient beaucoup plus long et qui absorbe une partie du matériel en excédent.

Plusieurs cas peuvent se présenter. Le péroné peut se terminer en pointe près de l'extrémité distale du tibia. Mais on observe aussi une tendance du péroné

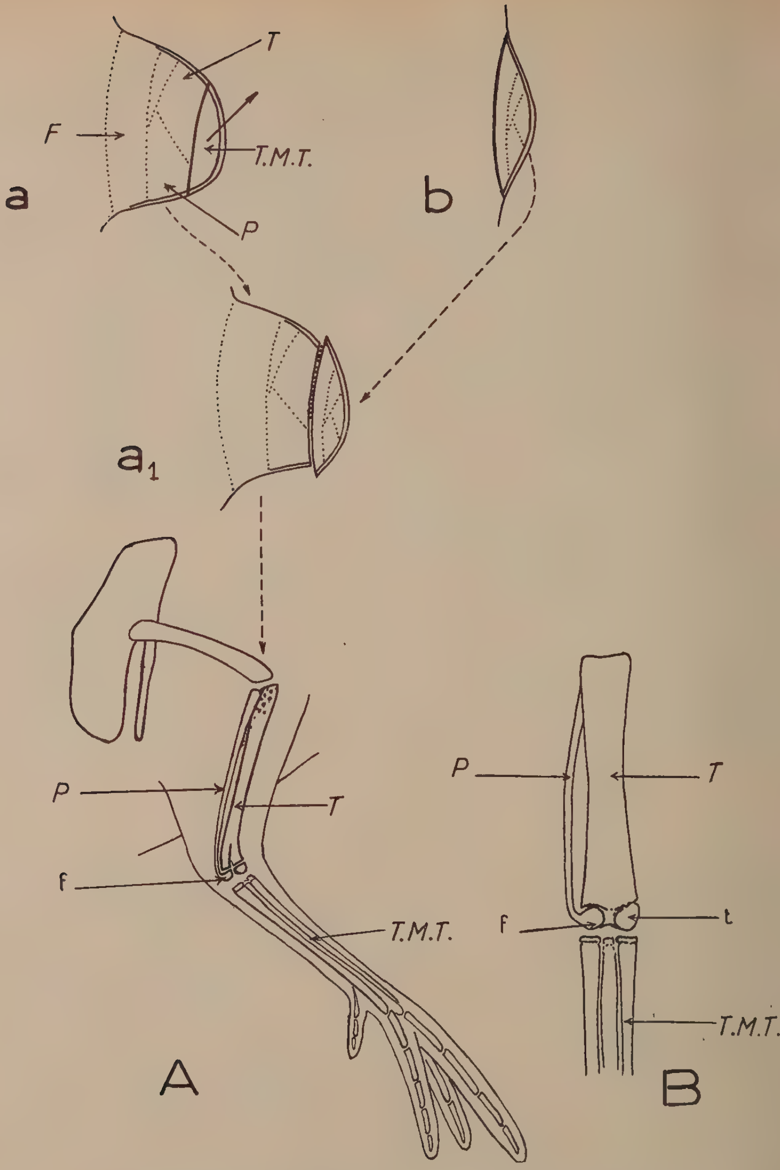

FIG. 2. La régulation des excédents. Formation d'un péroné complet.

Fig. *a*. Bourgeon de patte au stade 22. La partie distale, groupant le territoire présomptif du tarsométatarse, est excisée et remplacée par l'ébauche entière du bourgeon *b*.

Fig. a_1. Le moignon recouvert du bourgeon *b*. On voit la succession des deux séries d'ébauches.

Fig. A. Vue de profil de la patte évoluée dérivant du complexe a_1. Le péroné (*P*) très allongé se raccorde au fibulaire (*f*).

Fig. B. Le zeugopode vu de face. Le fibulaire (*f*) est raccordé au péroné (*P*); il continue cependant à former avec le tibial (*t*) la surface articulaire du tibia.

à se souder au fibulaire. On peut alors suivre le raccord qui relie le péroné et le fibulaire (fig. 2 A et B). Étant donné que ce dernier ne se trouve pas dans le prolongement normal du péroné, l'extrémité distale du péroné est contrainte de dévier pour rencontrer le fibulaire. On peut en conclure qu'il existe une affinité entre ces deux pièces osseuses.

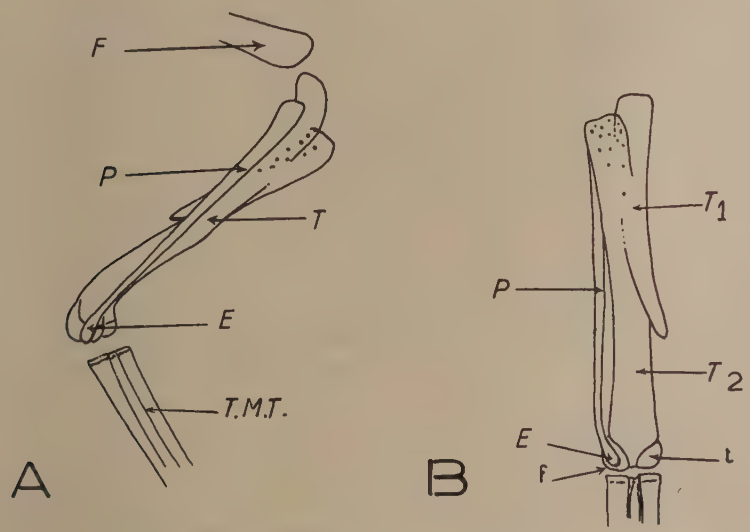

FIG. 3. Régulation des excédents. Autre cas de péroné complet.

Fig. A. Patte droite vue de profil. Le péroné (*P*), de même longueur que le tibia (*T*), est terminé par une extrémité distale élargie (*E*) qui est adhérente au fibulaire.

Fig. B. Patte droite vue de face. L'extrémité élargie du péroné (*E*) est appliquée au fibulaire (*f*). Le tibia (T_1) de l'hôte n'est pas entièrement fusionné avec le tibia du greffon (T_2). Par contre la constitution du péroné (*P*) est bien homogène.

Dans un autre cas, on peut même voir l'extrémité du péroné s'élargir et s'étaler contre le fibulaire (fig. 3). S'agit-il d'une épiphyse distale du pérone? La taille ne permet pas de le penser, on pourrait tout au plus en faire une ébauche, et sa disposition en contact avec le tarsien parlerait plutôt en faveur d'une zone de raccordement avec le fibulaire.

L'articulation du talon est donc formée, comme d'habitude, par les tarsiens mais, alors que, chez le Poulet normal, le tibia et le fibulaire sont soudés au seul tibia, le fibulaire est ici commun au tibia et au péroné, ce dernier participe par conséquent à cette articulation par l'intermédiaire du fibulaire.

DISCUSSION

Lorsqu'on compare le comportement du péroné en cas de pénurie et en cas d'excès de matériel, on est frappé par l'étonnante malléabilité de cette pièce. En cas de pénurie, le péroné est absent; en cas d'excès, il atteint la taille du tibia. Chez le Poulet normal, sa taille est intermédiaire, il se termine en pointe au milieu du tibia. On peut admettre que sa taille actuelle est incomplète; les lointains ancêtres des Oiseaux actuels possédaient certainement, comme l'Archéopteryx ou l'Archéornis, un péroné aussi long que le tibia. L'évolution ultérieure a amené une réduction progressive du péroné. Chez la plupart des Oiseaux actuels — c'est le cas du Poulet — la longueur du péroné ne dépasse pas les deux tiers de celle du tibia. Il existe cependant à l'heure actuelle des Oiseaux dont le péroné est moins réduit. Chez certains Rapaces (*Pandion carolinensis*) le péroné est presque aussi long que le tibia et soudé à celui-ci par son extrémité distale (Baur, 1885). Le même cas a été mentionné par Shufeldt en 1894 chez certains Sulidés (*Sula piscator* et *cyanops*). Le cas est identique à celui observé chez un autre Pélécaniforme, *Phalacrocorax*. Chez un Colymbiforme (*Colymbus septentrionalis*), l'extrémité distale et élargie du péroné est adhérente au tibia et atteint presque le fibulaire (Shufeldt, 1885). Ces différents cas observés sur des individus adultes correspondent à ceux que nous avons obtenus dans certaines de nos expériences sur la régulation des excédents, où le péroné est presque aussi long que le tibia, mais n'atteint pas le tarse.

Chez *Sula bassana* et *gossi* le péroné, soudé au tibiotarse à son extrémité distale, atteint la limite entre le tibia et le fibulaire. Chez *Fregata*, un Pélécaniforme, le péroné atteint le tarsien par son extrémité distale. Chez *Plotus auhinga*, appartenant au même ordre, le péroné se termine par une extrémité arrondie atteignant le fibulaire qu'il semble recouvrir légèrement. Ce dernier cas surtout ressemble à celui que nous avons obtenu et illustré par la figure 3. Le cas, décrit dans la figure 2, où le péroné se raccorde directement au fibulaire sans épaississement distal, n'a pas encore été observé chez les Oiseaux adultes actuels. Dans la série des péronés surdéveloppés, ce dernier cas se raccorde étroitement aux précédents et les continue. Ces différents types de péronés, observés sur des Oiseaux adultes ou obtenus expérimentalement, représentent vraisemblablement des stades intermédiaires entre l'Archéopteryx et le Poulet actuel.

Dans toutes les expériences ou observations précédentes, on peut conclure que le péroné a tendance à se rattacher au fibulaire. Cette affinité péroné-fibulaire, qui semble bien générale chez les Oiseaux, parle dans le sens d'un ancien rattachement entre ces deux pièces, rattachement qui ne pourrait plus se réaliser chez la plupart des Oiseaux actuels, par suite d'une réduction très précoce du territoire présomptif du péroné.

Chez les ancêtres des Gallinacés, la distribution topographique des ébauches était vraisemblablement un peu différente dans le bourgeon de la patte et le domaine péroné peut-être plus vaste. Dans le bourgeon de la patte actuelle, le

tibia s'accroît obliquement vers l'arrière et se déploie de plus en plus à l'avant du péroné. Ce dernier est ainsi peu à peu détaché du territoire des tarsométatarsiens. Il est possible que chez les ancêtres des Oiseaux actuels, les territoires présomptifs du tibia et du péroné se développaient parallèlement et avançaient de front vers les tarsométatarsiens.

L'évolution de la patte de l'Oiseau se ferait dans le sens d'une réduction progressive du territoire péroné, le stade actuel ne serait qu'un moyen terme sur le chemin de sa disparition complète. Cette réduction est compensée par le développement du tibia qui supporte à lui seul le poids du corps de l'animal. Le phénomène semble analogue à ce qu'on observe dans le zeugopode des Ongulés aux différents stades de l'évolution du groupe.

La malléabilité étonnante du péroné que l'on observe chez l'embryon d'Oiseau s'expliquerait par le fait que phylogénétiquement le péroné est en voie de transformation.

CONCLUSIONS

(1) Un bourgeon de patte de Poulet dont le tiers médian a été excisé se transforme dans les meilleurs cas en une patte normalement constituée, mais dépourvue de péroné.

(2) Lorsqu'on ajoute à un bourgeon de patte, amputé de sa calotte distale, un bourgeon plus jeune comportant les mêmes segments, on obtient une patte harmonieusement développée avec, en général, un péroné aussi long que le tibia.

(3) Le développement plus ou moins important du péroné semble lié à la quantité de matériel présent dans le bourgeon de membre.

SUMMARY

1. When an intermediate part of the limb-bud, amounting to one-third of its total length, is removed from a chick embryo at stage 20 of Hamburger & Hamilton (fig. $1a$, $1a_1$), the limb undergoes an almost complete regulation and grows normally, except for the fibula, which fails completely (fig. $1a_2$).

2. When a complete young limb-bud (stage 18–19) is grafted to another somewhat older limb-bud (stage 21–22) deprived of its apical part (fig. $2a$, $2a_1$), the resulting limb grows harmoniously, with all its normal divisions. An almost perfect regulation of the supernumerary material takes place, except for the fibula, which becomes abnormally long, of the same length as the tibia (figs. 2A, 2B, and 3).

3. The variable development of the fibula seems to depend upon the amount of material supplied by the limb-bud. A deficiency of material results in total failure of this bone, an excess of material evokes a complete fibula such as existed in the ancestors of birds.

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Regional Differences in Catheptic Activity in *Xenopus laevis* embryos

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INTRODUCTION

THAT the intracellular proteolytic enzymes known collectively as 'cathepsins' may have a special function in newly forming tissues was first suggested by the findings of Orechowitsch, Bromley, & Kusmina (1935). These authors observed that in certain amphibians regenerating tail-tissue had a much higher catheptic activity than the normal, non-regenerating tail. Their findings have been confirmed and extended recently in work on *Xenopus laevis* larvae, where it has been shown (Jensen, Lehmann, & Weber, 1956) that a peak in catheptic activity is reached in the regenerating tail-tip on about the 7th day after amputation. A further finding (Deuchar, Weber, & Lehmann, 1957) was that the early regenerate had a much higher catheptic activity relative to total nitrogen than had the stump-tissue immediately adjacent to it. The cathepsins seemed, therefore, to have the highest activity in the region of most active protein synthesis. But although Fruton *et al.* (1953) and Izumiya & Fruton (1956) have shown that under certain conditions *in vitro* a cathepsin may reverse its proteolytic action and catalyse synthetic steps, there is as yet no definite evidence that cathepsins ever catalyse protein synthesis *in vivo*. It remains possible, however, that some raw materials for the synthesis of new tissue proteins are provided by a prior proteolysis in which cathepsins play a part. Preliminary studies (Deuchar, Weber, & Lehmann, 1957) indicated that there was a rise in free amino acid concentration immediately after the rise in catheptic activity in the regenerating tail. But it was not proved that these amino acid increases resulted directly from protein breakdown.

During the early development of sea-urchin embryos, there are rapid fluctuations in free amino acid concentration (Kavanau, 1954) suggesting that the balance between rates of protein breakdown and synthesis is constantly changing. This is not surprising in an embryo that has no external source of nitrogen and therefore synthesizes much of its new protein during development at the expense of storage material such as the yolk. Associated with the breakdown of yolk and other proteins, one might expect also to observe a high

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catheptic activity in intra-embryonic tissues of most anamniote vertebrates, just as there is in the yolk sac of the chick embryo (Borger & Peters, 1933; Mystkowski, 1936). The evidence so far is scanty, however. Løvtrup (1955), Urbani (1955), and Vecchioli (1956) found only low catheptic activities in whole amphibian embryos, compared with larval stages. Some evidence of regional differences in the embryo is reported by d'Amelio & Ceas (1957), who find that in the early gastrula of *Discoglossus pictus* the dorsal lip has a higher catheptic activity than ventral tissue.

The dorsal parts of *Xenopus* embryos show, per unit dry weight, higher concentrations of free amino acids than ventral parts (Deuchar, 1956). Since this suggests that the relative rate of protein breakdown may be highest in dorsal parts, the catheptic activities of these same embryonic parts have been compared in the present work. The comparisons have been made first in absolute terms, then per unit dry weight (including fat) in order to compare them with the amino acid concentrations. But since the unequal distribution of yolk in dorsal and ventral parts greatly influences dry weight data (cf. Gregg & Løvtrup, 1950), the number of cells—and hence the catheptic activity per cell—has been estimated for each embryonic part as an alternative way of expressing the results. The catheptic activities of yolk and supernatant have also been compared after centrifugation of homogenates, as a possible clue to how closely linked the cathepsins are with yolk breakdown. Finally, an attempt has been made to characterize the cathepsins of these embryos more precisely by testing their reaction to cysteine, an activator of the mammalian cathepsins II and III (Bergmann & Fruton, 1941).

MATERIAL AND METHODS

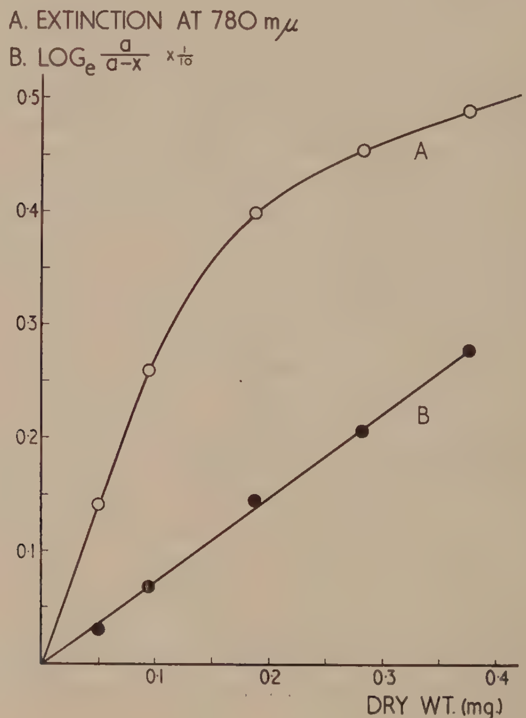
Preparation of samples. All glassware used in these experiments had been sterilized, and the salines and distilled water were autoclaved. *Xenopus* embryos were demembranated and dissected in Holtfreter's saline. Early gastrulae (stage 10 of Nieuwkoop & Faber, 1956) were divided into two parts: dorsal lip and remainder. Late gastrulae, early neurulae, late neurulae, and tail-bud stages (Nieuwkoop & Faber's stages 12½, 14–15, 19, and 25–26) were divided into dorsal and ventral portions by horizontal cuts opening the gut along its length. Gut-roof endoderm was therefore included in the dorsal part with axial mesoderm and neural tissue, while the ventral part included gut-floor endoderm and all the lateroventral mesoderm and epidermis. The embryonic parts were collected in groups of from 3 to 40 according to the experiment, transferred to glass-distilled water, then homogenized in the minimum of glass-distilled water in a ground-glass homogenizer on an ice-bath. Any material sticking to the homogenizer rod was washed into the tube with further distilled water, and at the same time the homogenate was adjusted to a convenient volume. For all volume adjustments and the taking of aliquots of homogenate or reagents,

Carlsberg-type constriction pipettes (Linderstrøm-Lang & Holter, 1933) with siliconed inner surfaces were used. As few as 3 embryonic parts homogenized in 100 μl. could serve as a sample for determining catheptic activity, but when dry weights were also to be estimated, larger samples (40 parts) were necessary.

Determination of catheptic activity. The method was based on that of Duspiva (1939), and has been described more fully by Deuchar, Weber, & Lehmann (1957). Determinations were made on duplicate aliquots of homogenate and these duplicates did not differ more than 2 per cent. in activity. The reaction mixture consisted of 33·3 μl. of homogenate with 112 μl. of a 1 : 1 mixture of 2 per cent. casein with phosphate-citrate-ammonia buffer solution (Duspiva, 1939). The pH of the mixture was 4·5 (preliminary experiments having shown that the catheptic activity was maximal at this pH). For convenience, a long incubation time of 18 hours at 38° C. was used. (A serum-broth test for bacterial infection, carried out on one set of samples at the end of the incubation period, proved negative.) 1,000 μl. of cold 5 per cent. trichloro-acetic acid (TCA) was then added to all tubes and they were left for at least 2 hours in the refrigerator to complete the precipitation of undissolved casein. After centrifugation at 3,500 r.p.m. for 25 minutes, a 1,000 μl. aliquot of each supernatant was taken. To this, 1,000 μl. of 13·5 per cent. sodium carbonate solution was added, followed by 1,000 μl. of Folin-Ciocalteu reagent, diluted 1/5. After $\frac{1}{2}$ hour the extinction value was read in a Hilger Uvispek spectrophotometer at 780 mμ, using distilled water as reference blank. Reagent blanks (casein/buffer with an aliquot of distilled water instead of homogenate) were incubated with each experimental series, and their mean extinction subtracted from all experimental values. In some experiments casein and homogenate blanks were also set up. These contained exactly the same mixture as experimental samples but had TCA added to them at once, and were not incubated. Since these blanks gave extinction values that were only 5 per cent. of the final experimental values, no extra correction was introduced except when homogenates of widely differing material were being compared.

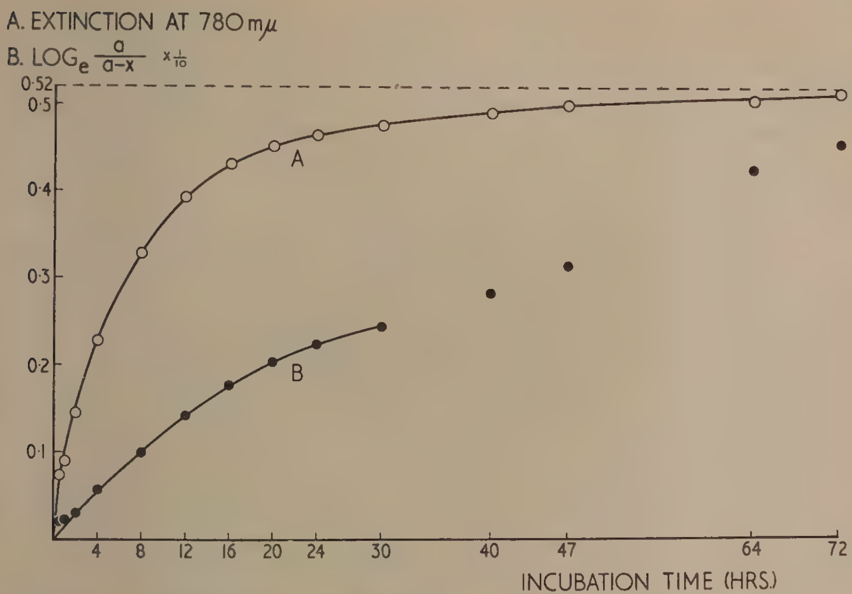
Expression of enzyme activity. A test with known dilutions of a standard homogenate of *Xenopus* late gastrulae showed that under the present assay conditions the extinction values at 780 mμ were not proportional to homogenate concentration except over a very narrow range of dry weight (Text-fig. 1A). Therefore for quantitative comparisons between different embryonic tissues and stages it was necessary to convert the spectrophotometer readings into other units that bore some proportionate relation to enzyme activity. The possibility that after so long an incubation as 18 hours the enzyme reaction would be inhibited by the accumulated products of casein hydrolysis or by other substances had also to be allowed for. The time-curve for a standard homogenate of late gastrulae (Text-fig. 2A) shows, in fact, a flattening as early as 8 hours, and tends towards an asymptote at the final extinction value of 0·52. Now when this terminal value 0·52 is substituted for *a* in the formula relating extinction

to time for a first-order reaction (viz. $kt = \log_e \frac{a}{a-x}$, where x = the extinction value at any time t) then a plot of $\log_e \frac{a}{a-x}$ against time for the same standard homogenate (Text-fig. 2B) is approximately linear up to 18 hours. The reaction thus behaves as though it were first-order up to this time and it would then be

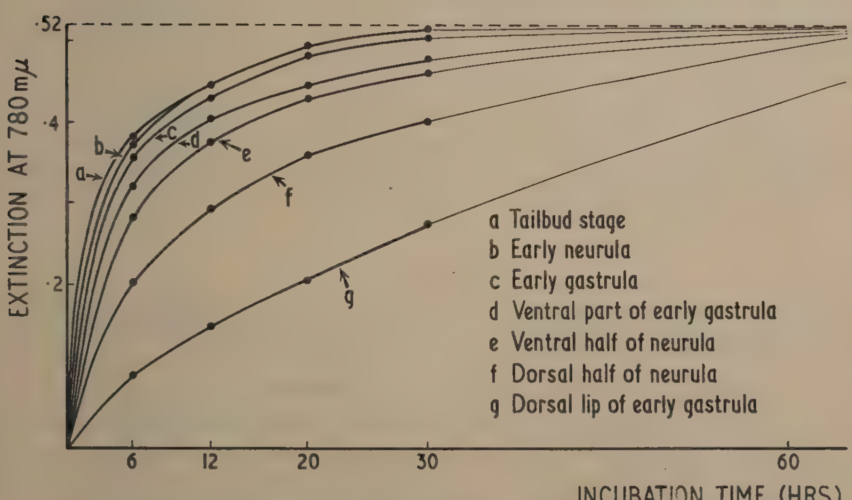


TEXT-FIG. 1. *A*: Extinction values at 780 mμ plotted against dry weight, for a standard homogenate of late gastrulae. *B*: $\mathrm{Log}_e \frac{a}{a-x}$ plotted against dry weight for the same homogenate ($a = 0{\cdot}52$; x = extinction value).

expected that a plot of $\log_e \frac{a}{a-x}$ against homogenate concentration ($a = 0{\cdot}52$ as above; x = extinction at any given concentration) would also be linear. Text-fig. 1B shows this to be true over a wide range for the data of Text-fig. 1. Making the assumptions that these findings hold for all homogenates, the value $\log_e \frac{a}{a-x}$ can be used as an 'activity value', i.e. a proportionate measure of enzyme activity. Since the time-curves for homogenates of a number of different



TEXT-FIG. 2. *A*: Extinction values at 780 mμ plotted against time, for a standard homogenate of late gastrulae. *B*: $\text{Log}_e \frac{a}{a-x}$ plotted against time, for the same homogenate during the first 30 hours ($a = 0{\cdot}52$; x = extinction value).



TEXT-FIG. 3. Extinction values plotted against time, for different embryonic stages and parts. (Data up to 30 hours only.)

embryonic stages and parts (Text-fig. 3) appear to tend towards the same asymptote at extinction 0·52, and since all the graphs of Text-fig. 4 are reasonably linear, the above assumption seems justified. All spectrophotometer readings (x) obtained in the experiments have therefore been converted into activity values by means of the same formula, and are quoted as such in the tables and figures.

Cell counts. The procedure of Sze (1955) was followed. Five embryonic parts were collected in 60 μl. of 1 per cent. citric acid in a microtube (capacity 300 μl.). After half an hour 60 μl. of 1 per cent. orcein in 45 per cent. (v./v.) acetic acid was added. The tissues were then broken up into free cells and nuclei with a glass rod fitting the inner dimensions of the tube. Care was taken to retrieve cells adhering either to the rod or to the walls of the tube, so that only negligible losses occurred. The number of cells and free nuclei was counted in 5 samples of the dispersion on a haemocytometer slide. At least 12 dispersions of each embryonic part were counted in the same way, and hence the mean numbers of cells per part were calculated.

Centrifugation of yolk. Whole neurulae were washed and homogenized in 0·28 M sucrose solution, buffered to pH 7 with M/15 phosphate buffer (Deuchar, 1953). After taking aliquots of the whole homogenate for determination of catheptic activity, the remainder was centrifuged at 3,500 r.p.m. on a semimicro angle centrifuge (Baird & Tatlock Ltd., London), for $1\frac{1}{2}$ minutes. This was the minimum time required to throw down a compact mass of yolk. The slightly cloudy supernatant was removed, and the yolk mass was washed twice by stirring it with 25 times its volume of buffered sucrose solution and recentrifuging. Aliquots of supernatant and yolk were used separately for assays of catheptic activity. (The volumes of original homogenate, supernatant, and yolk were adjusted so that each 33·3 μl. aliquot would be equivalent to the contents of one embryo.)

Dry weights. Known volumes of the homogenate remainders, after removal of aliquots for cathepsin assays, were transferred to weighing bottles and weighed on a balance accurate to 0·1 mg., after drying for 2 hours at 100° C. The dry weight per aliquot was then calculated.

EXPERIMENTAL RESULTS

Catheptic activity in whole embryos and parts of embryos compared in absolute terms

A homogenate aliquot equivalent to one whole embryo or one part was used for each assay of catheptic activity. The results (means of 5 determinations on whole embryos of each stage, and 10 determinations on each embryonic part) are given in Table 1.

Whole embryos. Late gastrulae showed a slightly higher catheptic activity than early gastrulae, and early neurulae a higher activity than late gastrulae ($P < 0{\cdot}01$ for both these differences). Late neurulae had, on the other hand, a

lower catheptic activity than early neurulae ($P < 0{\cdot}001$), then tail-bud stages showed again a slight increase in activity ($P < 0{\cdot}05$).

Parts of embryos. At all stages ventral parts showed a higher catheptic activity than dorsal parts. The difference was greatest in the early gastrula, and less marked but still significant in neurulae ($P < 0{\cdot}01$ for the dorsoventral differences in the late neurulae and tail-bud stages, and for all other stages $P < 0{\cdot}001$).

Catheptic activity per unit dry weight, compared between dorsal and ventral parts of the embryo

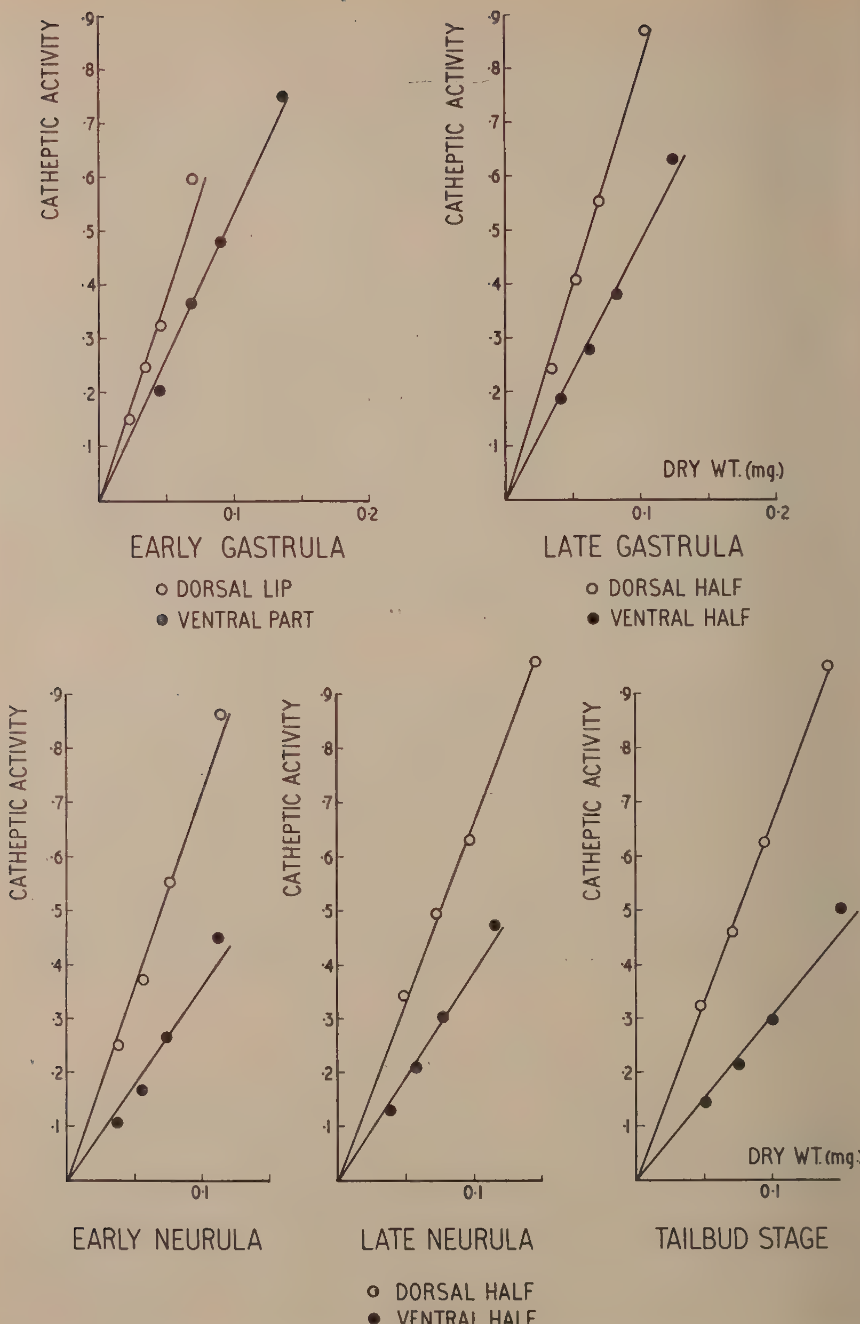
Each of the comparisons was made between homogenates of dorsal or ventral tissues over a series of different dilutions. Glass-distilled water was used as diluent, and homogenates of ventral parts were diluted 3 times as much as those of dorsal parts in order to obtain comparable ranges of dry weight in the two series. The results, plotted as graphs of activity values against dry weight (Text-fig. 4), show clearly that at each embryonic stage the activity per unit dry weight is highest in the dorsal part. Each pair of curves represents the results of only one experiment, but at least two repeat experiments were made for each stage, and the activity was always significantly higher per unit dry weight in the dorsal parts ($P < 0{\cdot}05$ for early gastrulae; $P < 0{\cdot}01$ for all other stages).

Catheptic activities per cell

The mean numbers of cells, estimated from counts of 12–14 samples of each embryonic part, are given in Table 1. As it was not possible to count cells in aliquots of the same homogenates used for cathepsin assays, only the mean cell numbers and mean activities could be used for estimates of the catheptic activity per cell. The resulting data (right-hand column of Table) are therefore only approximations since their variability is unknown. However, it is clear that except in the early gastrula the cell numbers are always highest in the *dorsal* part of the embryo ($P < 0{\cdot}001$), whereas the absolute catheptic activity, as already remarked, is always highest in the *ventral* part. Hence there can be no doubt whatever of the much higher catheptic activity *per cell* in ventral parts of the embryo.

Distribution of catheptic activity between yolk and yolk-free parts of the cells

Since the original volumes of homogenate, supernatant, and washed yolk platelets had been adjusted (see Methods) so that each $33{\cdot}3$ μl. aliquot was the equivalent of 1 early neurula's total contents, non-yolky contents, or yolk contents, the proportionate distribution of catheptic activity between yolk and other cell constituents is clear from a direct comparison of the activity units (bottom two rows of Table 2). It should be mentioned that haemocytometer counts showed the supernatant aliquots to contain, on an average, 500 small yolk platelets per μl., as compared with 70,000 platelets of all sizes per μl. in the precipitate aliquots. The good agreement between the data for different samples



TEXT-FIG. 4. Catheptic activity plotted against dry weight, for dorsal and ventral parts of embryos.

indicates that the centrifuging procedure, although very brief, was accurately reproducible. Reasonable agreement between yolk platelet counts on the various samples confirmed this. As Table 2 shows, there is clearly a much higher activity

TABLE 1

Catheptic activity and numbers of cells in whole embryos and dorsal and ventral parts

(For number of determinations per mean see text)

Stage	*Region*	*Catheptic activity**	*Number of cells†*	*Catheptic activity per cell* ($\times 10^5$)
Early gastrula	Whole	2·445±0·075	30,700±2,000	7·964
	Dorsal lip	0·455±0·030	16,100±1,200	2·826
	Ventral part	1·820±0·069	14,600±900	12·466
Late gastrula	Whole	2·828±0·076	52,600±1,700	5·376
	Dorsal half	0·663±0·051	32,400±1,200	2·046
	Ventral half	1·731±0·035	20,200±900	8·569
Early neurula	Whole	3·518±0·095	181,000±12,800	1·944
	Dorsal half	1·082±0·067	112,100±8,000	0·965
	Ventral half	1·577±0·091	69,000±5,100	2·286
Late neurula	Whole	2·740±0·093	184,300±12,000	1·487
	Dorsal half	1·128±0·083	118,400±7,900	0·953
	Ventral half	1·406±0·108	65,900±4,300	2·134
Tail-bud stage	Whole	3·334±0·223	207,900±13,700	1·604
	Dorsal half	0·908±0·060	136,400±9,330	0·666
	Ventral half	1·849±0·244	55,000±6,700	3·362

* Values for whole embryos obtained by a different set of assays from those on parts of embryos.

† Figures for whole embryos by summing individual data for parts of embryos.

TABLE 2

Distribution of catheptic activity between cell contents

(*A–E* are separate homogenates, each equivalent to one whole early neurula)

	Activity					*Mean and standard error*
	A	*B*	*C*	*D*	*E*	
Whole homogenate	1·776	1·800	1·776	1·835	1·721	1·782±0·031
Supernatant	1·382	1·356	1·360	1·454	1·375	1·385±0·016
Yolk	0·619	0·557	0·612	0·560	0·443	0·558±0·032

in the supernatant than in the yolk ($P < 0{\cdot}001$), but the activity remaining bound to the washed yolk is nevertheless considerable (about 40 per cent. of the activity in the supernatant). The data on whole homogenates are not comparable with those for early neurulae in Table 1 since they refer to different batches of eggs, but a preliminary experiment had shown that there was no significant difference

between the catheptic activities of comparable embryos homogenized in distilled water and in buffered sucrose.

Effects of cysteine on catheptic activity

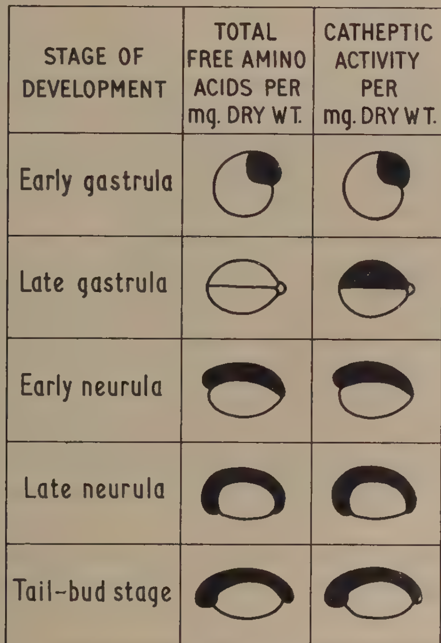
11·3 μl. aliquots of 0·033 M solution of cysteine-hydrochloride in Duspiva buffer were added to 33·3 μl. aliquots of homogenates of whole gastrulae immediately before incubating with casein / buffer solution. Hence the final concentration of cysteine-hydrochloride in the incubation mixture was 0·0024 M. The buffer solution had to be readjusted to produce the optimal pH of 4·5 as in all other incubations. Control aliquots of the same homogenates had 11·3 μl. of pure buffer solution, without cysteine, added to them. Blanks (distilled water instead of homogenate) both with and without cysteine were also incubated. The catheptic activity was much higher in the samples with added cysteine than in the controls. (Mean activity per homogenate sample was $0{\cdot}982 \pm 0{\cdot}050$ without cysteine; $2{\cdot}070 \pm 0{\cdot}150$ with cysteine added; $P < 0{\cdot}01$ for the difference.) The incubation time in this experiment was 4 hours.

DISCUSSION

Although Løvtrup (1955), Urbani (1955), and Vecchioli (1956) found only low and either constant or slightly decreasing catheptic activity in successive pre-tail-bud stages of whole amphibian embryos, the results presented here (Table 1) show that significant increases do occur. Since these are small in terms of extinction values, they may not have been detected by the earlier authors merely because their assays were adjusted to sensitivities suitable for the much higher catheptic activity in larvae. In the present results the increases of catheptic activity during gastrulation and neurulation are specially interesting since this is the time when the developmental fates of the embryonic tissues become determined and, in newt embryos, some new tissue-specific antigens appear (Clayton, 1953). So the rise in catheptic activity may have some relation to qualitative changes in the proteins. Since the only evidence we have on the function of cathepsins in *Xenopus* (Weber, 1957) suggests that they are proteolytic, they are probably best thought of in the embryo as mobilizing raw materials for synthesis, by the breakdown of pre-existing protein such as the yolk.

A striking feature of the regional differences in catheptic activity per unit dry weight is how closely they correspond (Text-fig. 5) to regional differences in free amino acid concentration (Deuchar, 1956). The late gastrula is the only exception, with a higher catheptic activity in its dorsal part, while there is no predominance of free amino acid here until the early neurula stage. This is reminiscent of the sequence of events observed in regenerating tail-tips of *Xenopus* larvae (Deuchar, Weber, & Lehmann, 1957), where high catheptic activity preceded increases in free amino acid. It may again be postulated (see Introduction) that the free amino acid increases result from proteolysis by cathepsins, but further evidence is needed to establish this.

Unfortunately it was not possible to measure concentrations of free amino acid per embryonic part or per cell, for comparison with the absolute catheptic



TEXT-FIG. 5. Diagrammatic comparison of amino acid concentrations and catheptic activity per unit dry weight, in dorsal and ventral parts of embryos. Black areas: significantly higher values than white areas of the same embryo.

TABLE 3

Concentration (expressed as extinctions at 510 mμ after ninhydrin reaction; Benz, 1957) of total free amino acid in dorsal and ventral parts of embryos

	Early gastrula	*Late gastrula*	*Early neurula*	*Late neurula*	*Early tail-bud*
Dorsal . .	0·145	0·113	0·264	0·124	0·130
Ventral . .	0·490	0·496	0·362	0·202	0·239

activities and activities per cell. But since all these ventral embryonic parts have at least twice the dry weight (including fat) of the corresponding dorsal parts, it was clear from earlier data that the total free amino acid per ventral part must, like the catheptic activity, be much higher than per dorsal part. A small additional experiment (results summarized in Table 3) has confirmed this estimate.

It follows that the free amino acid concentrations per cell are also, like the catheptic activity per cell, highest in the ventral parts of the embryos. This close parallel between the distributions of catheptic activity and free amino acid, whichever of these three different ways the data are expressed, lends support to the idea that cathepsins produce free amino acids in the embryo.

One remarkable general feature of the results is the complete inversion of dorsal/ventral differences according to whether they are expressed per unit dry weight or per cell and per part. Gregg & Løvtrup (1950) and Pickford (1943) have already demonstrated that quantitative comparisons between parts of embryos are greatly affected by the yolk content. They preferred to refer their data to 'non-yolk nitrogen' or 'extractable nitrogen' instead of to dry weight or total nitrogen, and other authors (cf. d'Amelio & Ceas, 1957) have followed suit. The idea underlying this preference is that the yolk not only contributes heavily to dry weight and total nitrogen, but is also relatively inert enzymatically. But this last contention can no longer be held, at least with respect to proteolytic enzymes. Harris (1946), Barth & Barth (1954), and Flickinger (1956) have demonstrated phosphoprotein breakdown in amphibian yolk, free of other cell constituents. In recent electron-microscope and solubility studies Gross & Gilbert (1956) have gone even further in demonstrating the complexity of yolk platelets, and speak of them as 'organelles'. Finally, in the present work the centrifugation experiments have shown considerable catheptic activity in the yolk fraction. The reference basis chosen for expressing quantitative data must depend on the particular viewpoint of each author. Here, while the data referred to dry weight show that dorsal parts of *Xenopus* embryos have the highest proportion of catheptic activity to total organic material, the data per part emphasize, in contrast, that there is, in sum, more catheptic activity in ventral parts. Finally, the *per cell* basis has been used because embryonic cells are undoubtedly important functional units, capable of much independent behaviour and differentiation. It has been specially interesting to demonstrate that ventral tissues, which are so often placed at the bottom of gradients in amphibian embryos that one has come almost to believe that they are in some way inferior, have in fact a higher catheptic activity per cell than dorsal tissues. A further interesting point emerging from the counts of cells is that the total cell numbers per embryo agree very closely with those in corresponding stages of *Rana pipiens* (Sze, 1953).

It is possible that the distribution of catheptic activity is related to the absolute numbers of yolk platelets in the cells and to the relative need for yolk breakdown. In chick embryos (Borger & Peters, 1933; Mystkowski, 1936; Emanuelsson, 1955) cathepsins are concentrated mainly in the yolk-sac, which is the chief site of yolk breakdown. In amphibians, however, the times and places of greatest yolk breakdown are not clearly known. It has been held (Bragg, 1939; Konopacki & Konopacka, 1926) that no appreciable breakdown of yolk platelets occurs until the late neurula stage, and that it then takes place mainly in the dorsal

tissues, but this view has never been supported by any clear quantitative observations. On the other hand, Daniel & Yarwood (1939) observed histological signs of yolk-breakdown as early as the zygote stage in newts. Moreover, recent electron-microscope studies (Yamada, pers. comm.; Bellairs, 1958) have shown in preneurula stages of newt and chick embryos some ultrastructural changes in the yolk platelets that may be the beginnings of their breakdown. It seems probable, then, that at the stages in which the catheptic activity has been studied here, some degree of yolk breakdown is taking place. The high proportion of catheptic activity remaining in the yolk fraction, even after thorough washing, suggests that in *Xenopus* the cathepsins are concerned in this process of yolk utilization. Some component of the catheptic system may even be an integral part of the yolk platelet.

It is known (Barth & Barth, 1954; Flickinger, 1956) that a phosphoprotein phosphatase plays an important part in yolk breakdown in *Rana* eggs, and it seems reasonable to believe that cathepsins act in conjunction with this enzyme. Casein, which is itself a phosphoprotein with many biochemical properties similar to vitellin, the predominant protein of yolk (Needham, 1931), is readily split by standard phosphatase preparations only after prior treatment with protease (Schmidt & Thannhauser, 1943). One might therefore suggest that yolk behaves like casein and that yolk breakdown takes place in two steps: first, a proteolysis carried out by cathepsins, and, secondly, a splitting off of phosphate by phosphoprotein phosphatase.

SUMMARY

1. The total catheptic activity per embryo in *X. laevis* increases during gastrulation and early neurulation, decreases slightly during late neurulation, then increases again at the early tail-bud stage.

2. When dorsal and ventral parts of gastrulae, neurulae, and tail-bud stages are compared, the catheptic activity per unit dry weight is highest in dorsal parts, but the absolute activity per part, and the activity per cell, are highest in ventral parts of the embryos.

3. The catheptic activity is increased in the presence of 0·0024 M cysteine-hydrochloride.

4. The yolk platelets, separated from other cell-constituents by centrifuging, carry a considerable proportion of the catheptic activity, though not as much as the supernatant fraction.

5. The possibilities that the catheptic activity gives rise to increased concentrations of free amino acids and that it assists in yolk breakdown are discussed.

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Type-specific Morphogenesis of Cartilages developed from Dissociated Limb and Scleral Mesenchyme *in vitro*[1]

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WITH ONE PLATE

THE emergence of definite organ and tissue structure during development implies that the component units—cells and intercellular materials—assume patterned space relations. These reveal themselves in geometric features of position, proportions, orientation, grouping, alignment, and so on. The ordering processes involved are variously referred to as 'organization', 'differentiation', 'morphogenesis', 'field action', and the like. Such terms, while useful as identifying labels for the respective problems, are largely lacking in concrete operational meaning. To realize this, one need only consider how little is known about the practical devices and tools by which any given product of development is achieved. The study of the 'biotechnology' of developmental mechanisms has indeed been lagging behind the preoccupation with general principles.

Development is a complex network of interlocking chains of processes. For an illustration of its intricacy we may refer to a diagram (P. Weiss, 1955, fig. 144) summarizing the results of efforts at resolving 'the development of the nervous system' into a series of more elementary events. To single out from such complex networks of relations simpler component threads thus becomes a prime prerequisite to a more penetrating factual analysis of developmental mechanisms. The following report offers a small contribution to such a programme, as applied to the problem of 'tissue architecture'; specifically, the architecture of cartilage as a prototype of a structurally simple tissue.

The objective of resolving histogenesis into component cell activities has recently been furthered by the introduction of procedures for the dissociation of tissues and of embryonic organ rudiments into their constituent cells and for

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the recombination of such cells into reconstituted tissues (Moscona, 1952, 1956). For demonstrations of the analytical possibilities of this approach see Moscona, 1957 *a*, *b*; Moscona & Moscona, 1952; Weiss & James, 1955; Trinkaus & Groves, 1955. To recapitulate the essential points: embryonic tissues can be dissociated by enzymatic action (trypsin) in such a manner as to free the individual cells in viable condition, whereupon these isolated cells, when allowed to aggregate *in vitro*, can reestablish tissue-like fabrics and, under appropriate conditions, give rise to typical differentiations in accordance with their original character. Evidently this method lent itself readily for the investigation of the problem of whether the architecture of a given skeletal unit was a composite result of contributions of the constituent cells, or something inherent to the organ rudiment as a whole.

Cartilages differ characteristically by their shapes, which in turn are but indices of the differential patterns of their antecedent growth and differentiation. Growth patterns, in turn, are resolvable into more elementary component features: the configuration of the original cell population of a blastema; secondary regroupings; differential rates of proliferation, resulting in unequal expansion and crowding of different regions; different rates of production, deposition, and swelling of intercellular ground substances; confinement, creating internal strains; and passive deformation by external stresses. External factors of the latter sort definitely cannot account for the basic differences of growth pattern that distinguish different skeletal elements. For in tissue culture, under otherwise identical conditions, various precartilaginous blastemas continue to grow into different shapes corresponding essentially to their sites of origin. Thus, explanted precartilaginous mesenchyme from the limb-bud of a chick embryo turns into parts of a typical limb skeleton (Fell & Canti, 1934; Niven, 1933); while explanted mesenchyme from the ventral midline forms a sternum with its diagnostic median keel (Fell, 1939). Similarly, precartilaginous mesenchyme of the coat of the eye in culture gives rise to a flat plate characteristic of scleral cartilage (Weiss & Amprino, 1940). Although the eventual shapes to which these different growth patterns give rise vary in morphological details according to the nutrient conditions (Fell & Mellanby, 1952) and mechanical stresses (Glücksmann, 1941; Weiss & Dorris, 1936; Weiss & Amprino, 1940), which have prevailed during their gradual elaboration, their basic architectural features are already pre-established in the mesenchymal blastemas at the time of their explantation.

Here, then, are tissue fragments which, in a common medium, take conspicuously divergent developmental courses and which, because of the relative simplicity and geometric definition of their eventual distinctions, seemed to be singularly suited for the next step of analysis—the identification of critical differentials among the different chondrogenic blastemata. Gross microscopic inspection has furnished no clue. Since the most obvious distinctions are those of shape—that is, of the particular patterns in which the cells are arranged—and

since the positions of the cells in the group are relatively fixed by the cementing action of the common ground substance, however tenuous at first, it seemed most plausible to look to the intercellular matrix as the seat of the critical structural differences. In all the experiments referred to above, the embryonic fragment had been removed and transferred into culture in one piece, thus preserving whatever internal architecture might have existed in it at the time. But whether or not this preservation of group integrity, as a patterned entity, was actually relevant for the subsequent pattern of development, had remained undecided. For a crucial test of this question, one had to go further and disrupt the inner organization of the blastemas thoroughly prior to explantation, as had become feasible by the trypsin dissociation method. It had been established that disassociated and reaggregated mesenchyme cells from the embryonic chick limb-buds formed compact nodules of cartilage bearing the typical cytological and histological characteristics of chondrified tissue (Moscona, 1952, 1956; Trinkaus & Groves, 1955). But whether such pieces, beyond becoming just generalized cartilage, also assumed definite morphological features, indicative of their origins, could only be decided by further experiments, comparing the differentiation *in vitro* of cells from dissociated blastemas of two different cartilage types of grossly divergent architecture. For this we chose scleral cartilage with its sheet-like growth to be contrasted with limb cartilage with its massive expansion.

MATERIAL AND METHODS

The experiments were done with cells from chick embryos. Since appendicular cartilage develops earlier than scleral cartilage, test mesenchyme of limb-buds was taken from $3\frac{1}{2}$- to 4-day embryos, that of eye coat from 6- to 7-day ones. If explanted at these stages *in toto*, the former would give rise to limb-like cartilages (Fell & Robison, 1929; Fell & Canti, 1934), while the latter would form scleral plates (Weiss & Amprino, 1940). The former is characterized by essentially three-dimensional growth centres, with the cells assuming the configuration of concentric whorls; while the latter grows as a plate of uniform thickness, normally about 3–4 cell layers in width, bounded by smooth parallel surfaces and showing in its interior heavy fibrous tracts in planes parallel to the surface, which give it an appearance of abortive lamination.

The rudiments were removed under the dissecting microscope and dissociated into the component cells by the trypsin procedure, as previously described (Moscona, 1952, 1956). Plate, figs. A, B, show fixed and stained smears of cell suspensions from the two types of chondrogenic rudiments. There were no perceptible microscopic differences between the individual cells of the two types after isolation. The suspended cells were then allowed to settle and to aggregate in the concavity of a Maximow slide containing culture medium consisting of equal quantities of chick serum (Difco), fresh chick embryo extract (10–12 days), and Earle's balanced salt solution. The aggregating cells formed cohesive

clusters or sheets. These formations were removed after 24 to 48 hours from the liquid medium and transferred to the surface of clots of chicken plasma and embryo extract mixed in a ratio of 3 : 1. Each culture was set up to contain only one of the two types of mesenchyme tested and no mixtures have been tried in these experiments.

The cultures were fixed after 2 to 5 days *in vitro*, sectioned perpendicularly to the clot surface, and stained with hematoxylin-eosin or with the periodic acid-Schiff reagent.

RESULTS

Already during the phase of aggregation in the liquid medium, a marked difference in the behaviour of the two types of cells became noticeable: while the limb-bud cells, as described previously (Moscona & Moscona, 1952), aggregated into numerous separate clusters, each of which formed a rounded nodule, the prescleral cells usually settled in a single continuous sheet coating the bottom of the slide. These configurations thus seemed to anticipate the basic morphologies of their later products. During further cultivation on plasma clot, both cell types developed into histologically typical cartilage. Morphologically and structurally, however, these cartilages were distinguished from each other by the possession of features referrable to their original prospective architecture. Thus, cartilage derived from limb mesenchyme appeared as scattered nodules, irregularly shaped, with cells arranged in concentric whorls around numerous foci (Plate, figs. C, G). By contrast, cartilage derived from prescleral cells had formed smoothly bounded plates in which the cells were predominantly arranged in planes parallel to the surface, interspersed with fibre systems in the ground substance of corresponding parallel orientation (Plate, figs. D, F). The results thus point to the conclusion that the individual cells of the two types of blastemas had already been constitutionally different at the time of their isolation and had each contained properties which would enable them to elaborate their own appropriate tissue architecture when reassembled in a group.

Before accepting this conclusion, however, an alternative explanation had to be ruled out. As mentioned above, the limb-cell aggregates had already rounded up, and the prescleral cell cultures had assumed the shape of a sheet, during the aggregation phase. This initial difference in arrangement was essentially preserved as they were transferred on to the clots for cultivation. Now, it is known that external shape determines internal stress patterns which, in turn, affect the disposition of cells and ground substance (Weiss & Dorris, 1936; Weiss & Amprino, 1940); and since in these tests we had to rely on cellular configurations as major criteria, differences in the constellation in which the aggregated cells were initially placed on the clot had to be taken into account. Conceivably, chondrogenic mesenchyme, from whatever source, might give rise to either massive cartilage of the limb type or to flat plates resembling sclera, depending

on whether the aggregated cells be cultured in a solid, rounded clump or in a flat sheet.

To check this possibility, the experiments were repeated with a deliberate effort to make the geometrical conditions during the culture phase more nearly alike for both types of aggregates. This was done as follows. In one set, dissociated limb-cells in a very thick suspension were poured on to the plasma clot surface as a dense, continuous layer, thus imitating the spontaneous layering typical of prescleral cell aggregates formed in liquid culture medium. In the other set, newly formed sheets of prescleral cells, instead of being spread on the clot, as in the preceding experiment, were bunched so as to be given the lumpy shape and dimensions of a limb-cell nodule.

The two types of cartilage developing under these more strictly comparable conditions still exhibited the constitutional differences of their respective architectures. Limb-cells plated out as a sheet gave rise to cartilage that had externally the dimensions of a plate, but internally the characteristic whorl-shaped texture of typical limb cartilage aggregates (Plate, figs. E, G), contrasting with the laminar texture of scleral cartilage developed under the same conditions (Plate, figs. D, F). Reciprocally, prescleral cells, cultured as massive lumps, still produced plate-shaped cartilage, albeit bent and folded inside the lump because of the spatial confinement of the building material (Plate, fig. H). In other words, regardless of whether the initial distribution of the explanted material on the clot favoured essentially planar expanse or more massive clustering, the way in which the cells filled the allotted space, grouped themselves, proliferated, and deposited intercellular ground substance, was determined by the properties of the particular cell type; evidently, the individual cells of either type must have been already endowed with those properties at the stage when they were separated. These structural differences are perhaps more obvious when limb cartilage is allowed to develop *en masse*, and scleral cartilage as a sheet, each type being thus free to assume a shape conforming to its intrinsic growth pattern. Yet, even in the case of an artificially created inconsistency between the growth and grouping tendency of these cells and the external configuration of the growing mass, the intrinsic growth and group patterns still prevail.

DISCUSSION

The foregoing results lead to the following conclusions. Different chondrogenic blastemas, destined to form specific parts of the skeleton, consist of cells which, at the stages tested, are endowed not only with a general faculty to turn into cartilage cells, but furthermore with distinctive morphogenetic properties determining the particular patterns of cell grouping, proliferation, and deposition of ground substance which, in due course, lead to the development of a cartilage of a distinctive and typical shape. This implies that the crucial morphogenetic properties in question reside in the individual cells, for whether or not their assembly—the blastema—retains its original structural integrity has

proved to be quite immaterial for the subsequent morphogenesis. Even after the complete disruption of the chondrogenic blastema and the random reassociation of the separated cells, the latter still know how to build that same typical cartilage.

Whatever this remarkable property be, it cannot manifest itself, of course, in single cells and is evidently a group phenomenon. A single cell can form neither a plate nor a whorl. The property in question, therefore, must be of a sort that would enable the individual cells, when they join together with others equally endowed, to execute collectively a group operation of a higher degree of orderliness. Supercellular self-ordering processes of this kind conform to the original definition of 'field' effects (P. Weiss, 1923; summarized in P. Weiss, 1939, 1953). It is a great step forward to have come to the realization, from experiments such as here reported and similar previous ones (Moscona & Moscona, 1952; Grobstein, 1952, 1953; Andres, 1953; Weiss & James, 1955), that 'fields' may originate by active integration of formerly independent cells, or cell groupings, of given types congregating at random. At the same time, one must not lose sight of the fact that the different cell types endowed with these differential faculties for specific group performances have contracted them during earlier phases of their embryonic history while they themselves resided within integral field districts. In its general features, the phenomenon of field reconstitution from random dispersed cells resembles closely the morphogenesis of the fruiting bodies of slime moulds achieved by a collective action of populations of free amoebae (cf. Bonner, 1951); it also validates, in a purely formal sense, some of the principles of morphogenesis envisaged by Child (1941), though not necessarily his particular assumptions as to the mechanisms involved.

Which brings us just to the crucial question of mechanism. The fact that dispersed and reaggregated epidermal chick-cells can constitute a feather field *de novo*, even in tissue culture (Weiss & James, 1955), though theoretically of interest, still tells us nothing of just how the reassembled cells go about accomplishing that feat. Perhaps the reproducibility of the process under the simplified conditions *in vitro* will help elucidate it; but this is still a matter for the future. The same is true of our present cartilage case. Besides telling us that some cells know how to build a plate, and others a nodule, the experiments have yielded no safe clue as to how they do it. However, since cartilage structure is considerably simpler than feather structure, this kind of tissue may provide a more favourable object for a deeper penetration into the mechanisms of structure formation, referred to in the introduction as 'biotechnology'. The next step should be to detect more elementary differences in behaviour of cells which preferentially form plates versus cells which tend to form lumps: differences in aggregation, in mutual orientation, in proliferative pattern, and perhaps in the fine-structural characteristics of their secreted ground substances. In view of the fact that some extracellular fabrics have recently been intimated to possess 'pseudo-crystalline' organization (Weiss & Ferris, 1956), it is not implausible to

conjecture that the ground substances of the cartilage may likewise play a unifying and structure-determining role, the cells thus generating an ordered matrix, to the ordering influences of which they themselves would then reciprocally submit (P. Weiss, 1933).

Although these experiments have been the first to establish explicitly that morphogenetic distinctions between cartilages are based on properties of their individual constituent cells, rather than on a property of the blastema 'as a whole', it may be worthwhile to point out that this outcome might have been actually suspected long ago from observations on the development of branchial cartilage in amphibians, which takes its origin from the distant neural crest. Crest material contributes circumscribed portions to the head skeleton, mosaic-fashion (Stone, 1929; Hörstadius, 1950). The cells concerned migrate in streams from their dorsal positions in the neural crest to their peripheral destinations and, to all appearances, they are not firmly assembled during this trek. How, then, do they manage to build a particular skeletal element, e.g. a palatoquadratum, once they have aggregated again at their final site? One might have invoked a moulding role of 'organizing' factors of the local environment were it not for the fact that in xenoplastic combinations (e.g. frog hosts provided with toad neural crest) the foreign cells, even though under general guidance from the host, form the appropriate skeletal pieces strictly according to the rule of their own species-specific pattern (Wagner, 1949). Since the swarming from the neural crest can be legitimately compared to experimental dissociation, it would seem equally valid to consider the orderly group performances after reconcentration and aggregation as essentially of the same nature—and equally remarkable—in both cases. The origin of the limb-bud from freely migrating mesenchyme of the somatopleura in amphibians (Balinsky, 1929; Taylor, 1943) may also have to be reconsidered in this light.

SUMMARY

1. Precartilaginous blastemas of chick limb-buds ($3\frac{1}{2}$–4 days) and chick sclera (6–7 days) were dissociated into their constituent cells; the cells from each type were allowed to settle and to reassociate in a liquid tissue culture medium, and the newly established groups were cultivated on plasma clots *in vitro*. Both types of cells produced cartilages, yet each according to the specific pattern characteristic of its origin: limb mesenchyme formed lumpy nodules with whorl-shaped cell arrangements, which eventually gave rise to masses of cartilage similar to that found in normal appendicular skeleton; whereas prescleral mesenchyme formed flat plates with pseudostratified texture, much as normal scleral cartilage.

2. Either cell type maintained its intrinsic structural characteristics even when the explanted cell masses were experimentally given external shapes corresponding to the other type (limb-bud cells by layering on plasma clot and prescleral cells by compacting, respectively).

3. The individual cells of each cartilage type were thus shown to have possessed at the time of their dissociation distinctive properties, including a type-specific rule of assembly and subsequent growth which enabled them eventually to elaborate collectively a supercellular architecture of the same type as that which they would have formed if they had been left undisturbed.

4. The phenomenon described may be regarded as a simple instance of a 'field' effect and as such lends itself readily to further experimental analysis.

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EXPLANATION OF PLATE

FIG. A. Suspension of disassociated chondrogenic cells from the limb-buds of a 4-day chick embryo. (Ehrlich's hematoxylin, Biebrich's scarlet. ×760.)

FIG. B. Suspension of disassociated chondrogenic cells from the prescleral mesenchyme of the eye coat of 6½-day chick embryos. (E.h., B.s. ×760.)

FIG. C. Clusters of aggregated and differentiated limb cartilage cells, grown *in vitro* for 5 days. Notice the concentric or whorl-like arrangement of cells in the differentiated and chondrified masses. (E.h., PAS. ×200.)

FIG. D. Scleral cartilage formed by aggregated prescleral cells. Following aggregation in a liquid culture medium, the cellular sheet was transferred to a plasma clot and cultured for 5 days. (E.h., B.s. ×80.)

FIG. E. Limb cartilage formed by disassociated limb-bud cells spread on a plasma clot to encourage sheet-like development. Five-day culture. Notice the whorl-like clusters and compare the structural appearance of this tissue with the scleral sheet in fig. D. (E.h., B.s. ×80.)

FIG. F. Reconstituted scleral sheet in culture, similar to that in fig. D, magnified to show the architecture of the tissue. (E.h., B.s. ×800.)

FIG. G. Limb-bud cartilage, reconstituted in sheet-like form, similar to fig. E, magnified to show the architecture of the tissue. (E.h., B.s. ×800.)

FIG. H. Culture of aggregated scleral cells. The sheet formed by these cells in the liquid medium was bunched so as to give this aggregate the shape of a nodule which was then grown on a plasma clot for 4 days. Notice the presence of a folded, sheet-like, scleral structure within the clump. Due to their size these explants frequently showed considerable necrosis. (E.h., B.s. ×120.)

FIG. I. Part of a reconstituted scleral sheet grown *in vitro* for 7 days. In spite of marked growth and expansion, the characteristic shape of the tissue was retained. (E.h., B.s. ×30.)

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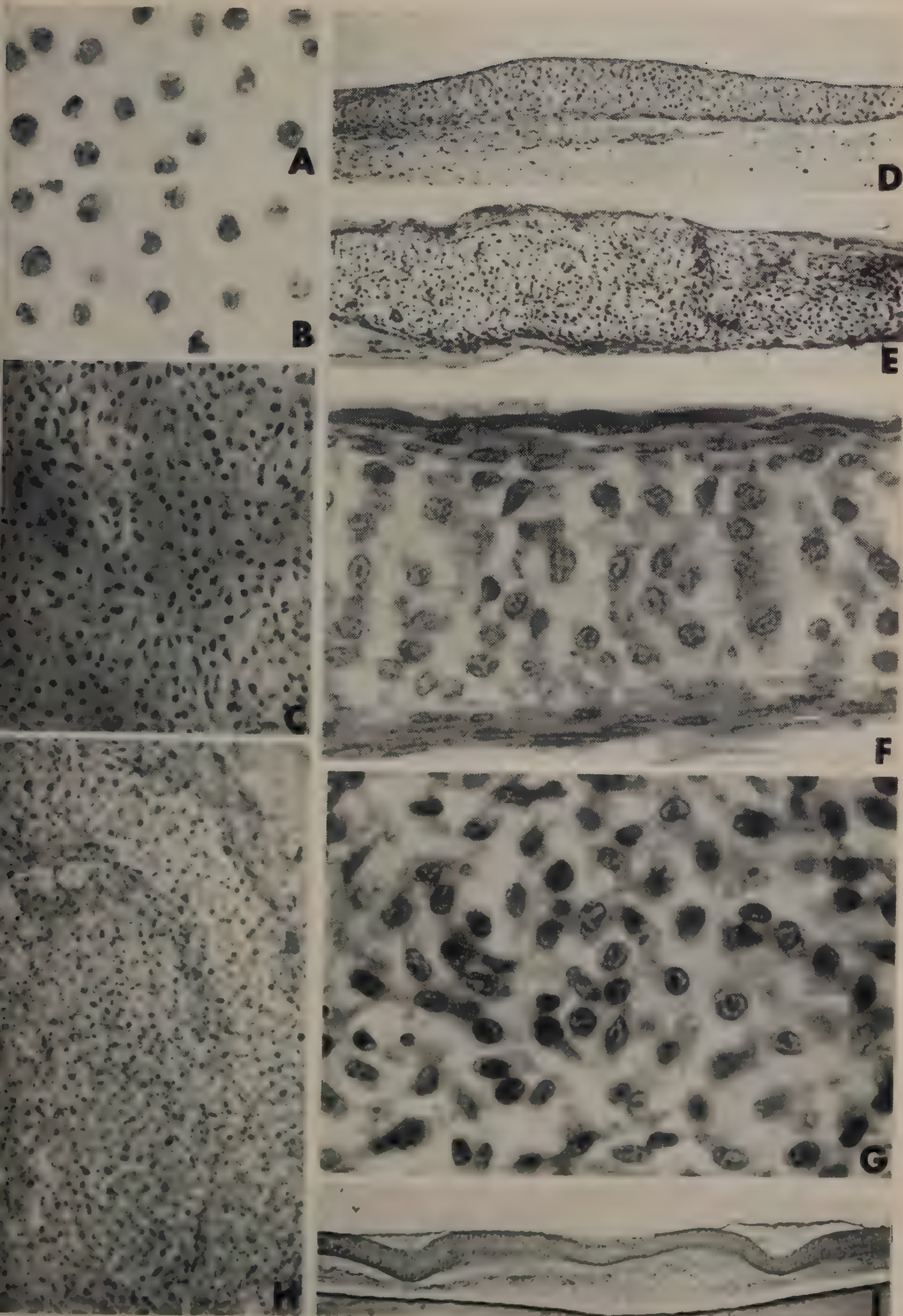

P. WEISS and A. MOSCONA

Effects of Adrenal Transplants upon Forelimb Regeneration in Normal and in Hypophysectomized Adult Frogs

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WITH FIVE PLATES

INTRODUCTION

THE frog provides among the vertebrates the best opportunity to investigate the factors which determine loss of regeneration within the ontogenetic boundaries of a single organism, for in tadpoles the capacity to regenerate tapers off along the proximo-distal axis of their limbs and in adult frogs limb regeneration is substantially absent.

It was generally accepted that metamorphosed frogs lose this capacity because of progressive complexity of structure in the growing limbs (Marcucci, 1916; Polejaiev, 1936; and Forsyth, 1946). However, the conclusion that it is the nature of the limb tissues which determines presence or absence of regeneration was shown to have only limited validity when regeneration was obtained in metamorphosed frogs. Thus Polezhajev (1945) demonstrated that repeated trauma to the amputational surface of limbs of adult frogs (which according to his own theory had irrevocably lost the powers of regeneration because of progressive differentiation) was sufficient for recuperation of this faculty in a substantial number of cases. By immersing metamorphosed frogs in hypertonic salt solutions, Rose (1944, 1945) obtained limb regeneration; and finally, Singer (1954) (and indirectly also Van Stone, 1955), by increasing the nerve-supply of limbs, obtained regeneration from normally unresponsive amputational levels.

The papers of Walter (1911), Schotté (1926), Richardson (1940–5), Hall & Schotté (1951), and Schotté & Hall (1952) concurred in showing that both the pituitary and the thyroid glands influenced regeneration, but these findings could not explain the cessation of regeneration in Anura (Naville, 1927; Guyénot, 1927; and Schotté & Harland, 1943).

The suspicion that cellular properties did not play so dominant a role in regeneration as the above evidence seemed to suggest, became a near-certainty

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when the endocrinological factors in regeneration were re-examined in the light of discoveries made by mammalian endocrinologists in regard to the pituitary-adrenal synergism under stress conditions (Selye's general adaptation syndrome, 1947; Selye & Stone, 1950). In 1952 Schotté & Hall proposed that, in a manner already known from mammalian endocrinology, the role of the pituitary in urodele regeneration was probably confined to stimulation of the adrenal cortex after amputational stress. The reality of a pituitary-adrenal synergism in respect to regeneration was demonstrated when, in newts deprived of their pituitary, replacement therapy with ACTH (Schotté & Chamberlain, 1955) and with cortisone (Schotté & Bierman, 1956) restored the regenerative capacities in these animals. The direct involvement of the adrenals in regeneration of urodeles became a certainty when Schotté & Lindberg (1954) induced regeneration in hypophysectomized newts by transplantations of frog adrenals.

Because of marked similarities between urodele and anuran regeneration, and because of the well-known involvement of endocrines in metamorphosis, a process which seemed to coincide with loss of regenerative processes in Anura, it became imperative to determine whether artificial changes in the endocrine system of frogs could modify regeneration of their limbs. The first attempts to investigate this problem were made a few years ago in this laboratory when it was shown that the transplantation of additional adrenals could lead to restoration of regenerative capacity in premetamorphic tadpoles at normally non-regenerating amputational levels (Lindem, unpublished thesis, Amherst College, 1954).

The realization that lost regenerative ability was regained in tadpoles by inducing a hyperadrenal state offered an irresistible invitation to conduct still further investigations on adult frogs. The purpose of this research, therefore, has been to determine whether or not the normally absent capacity for limb regeneration in adult frogs could be recovered by introduction of additional adrenal glands into these animals.

MATERIALS AND METHODS

The effects of adrenal transplants upon the regeneration of forelimbs were studied in two American species of frogs: *Rana clamitans*, commercially procured from the south of the U.S.A.; and *R. pipiens* (donor of all the adrenal glands), secured from Wisconsin and northern Vermont. These postmetamorphic frogs were force-fed twice weekly on beef-liver with bone-meal supplement before and after the operations. For all the operations the frogs were narcotized by immersion in a solution of MS 222 (1 : 1,000), a meta-amino-benzoic acid ethylester in the form of methanesulphonate (Sandoz, Basle).

Amputations were made at either the mid radius-ulnar level or at the distal humerus level of the frog forelimb. These amputations were performed under a dissecting microscope, and, immediately after the initial severance, the pro-

truding bone elements were re-amputated to adjust them to the level of the retracted soft tissues.

For transplantation experiments both narcotized host and donor frogs were placed in petri dishes upon sterile surgical gauze moistened with sterile physiological salt solution. The adrenal glands in frogs appear as thin ribbons of a golden yellow colour extending along the median ventral surface of each kidney. The donors' kidneys were dissected out and placed upon surgical gauze in another sterile dish and the adrenals were excised from the kidneys with iridectomy scissors and Swiss watchmaker's forceps underneath a binocular microscope. Care was taken to minimize the amount of kidney tissue adhering to the adrenal bodies. All transplantations were made into the lower jaw because this region of the frog is highly vascularized. For the insertion of the prepared adrenals a small opening was made in the skin of the lower jaw with iridectomy scissors. A sterile probe was used to enlarge the subcutaneous pocket into which the pieces of adrenals were pushed as far anteriorly as possible to prevent post-operative extrusion; no transplants were lost.

The pituitary gland in the frog is easily identified through the semi-cartilaginous parasphenoid bone. The operative procedure was adopted from Levinsky & Sawyer (1952), with the difference that we extirpated the exposed pituitary with watchmaker's forceps instead of with a pipette.

After any surgical procedure the animals were kept for one day at 13° C. (±1° C.) in finger-bowls, the bottoms of which were covered with surgical gauze and moistened with sterile physiologic solution to which a small quantity of sodium sulphadiazine was added. After 1 day the animals were transferred into demineralized water, about ten per aquarium, and were maintained at 20° C. (±1° C.) for the duration of the experiment.

This research is based upon a study of sixty-five surviving animals from a much larger number of operated frogs. From these cases forty-seven forelimbs were studied histologically; and in addition, the lower jaws of five frogs were sectioned serially to investigate the condition of the adrenal transplants especially in regard to vascularization; also, the heads of six hypophysectomized animals were examined in sections to verify the completeness of pituitary removal. The tissues were fixed in Bouin's, decalcified in Jenkin's solution, stained with Harris's modification of Delafield's hematoxylin and counterstained with orange G. For the study of slides of adrenal tissues the sections were stained by Mallory's polychrome method.

OBSERVATIONS AND RESULTS

1. *Survival and functional state of adrenal transplants*

At various times the conditions of forty-five heteroplastic or homoplastic adrenal transplants were studied among the fifty-three frogs having received similar transplants. For gross study the whole subcutaneous area of the lower

jaws was exposed by dissecting away the skin and in all cases the transplants were found embedded within the richly vascularized sub-dermal tissues. Examination of these transplants *in vivo* under the dissecting microscope and study on slides both concur to show that the transplanted adrenals remained viable, normally pigmented, and fully vascularized for as long as 125 days, the longest period examined.

The colour photograph of Plate 1 illustrates the survival and the state of vascularization of two consecutive heteroplastic transplantations of adrenal glands from *R. pipiens* cut in both instances into four pieces to enlarge the surface area. (See explanation of plates for further details.)

The amount of tissue diminishes with time and gradual histolysis was unmistakable; but fragments fixed for histological study around 30 days after the last transplantation appeared normal and healthy: the frog erythrocytes were easily identified within the blood-vessels connecting the host's tissues with the heterotopic adrenal bodies. The cortical elements appeared as oblong groups of cells and anastamosing cords, separated by blood sinuses, and this aspect complies in all respects with the histological description of anuran cortices given by Jones (1957). Therefore, from both gross and histological evidence it is inferred that the adrenals *ex situ* were alive and received an adequate blood supply during the period considered. These indices of healthy survival of adrenocortical elements suggest, but do not prove, the functional activity of these tissues. Few of the many physiological effects of the hyperadrenal state indicated in the literature (Selye & Stone, 1950) have been detected in this study.

No visible atrophy of the adrenals was observed in normal frogs with the exception of pigmentation losses in cases with large dosages of adrenal transplants. Cameron (1953) described peripheral stasis of small blood-vessels and the presence of mucous exudates as symptomatic of a hyperadrenal state, but neither of these conditions was seen in these experiments. Our observations would suggest that the adrenal transplants were not acting cumulatively.

2. *Morphological and histological effects of forelimb amputations in control frogs* (24 postmetamorphic *R. clamitans*; mean body length from nose tip to cloaca, 4·7 cm., range, 4·2 to 5·7 cm.; 16 cases studied histologically)

(*a*) *Simple amputations* were performed in ten cases to study the histological aspects of non-regeneration in adult frogs, since no such investigations are known to us from the literature. Singer (1954) confines himself to descriptions of the morphological features of early cicatrization in amputated forelimbs, and we concur fully with his descriptions.

Limbs were amputated either through the forearm or the upper arm and fixed at intervals between 15 to 50 days after amputation. No differential rate of cicatrization was noted between the two amputational levels and this is fully confirmed by slides. Two sections (one for each level) are presented: Plate 2, fig. A represents the usual features of non-regeneration in a longitudinal sec-

tion of a limb amputated through the radius-ulna: the wound surface is completely healed with epidermis and dermis containing newly formed skin glands covering the amputation surface; directly beneath lies the conspicuous and uninterrupted basement membrane. Another feature that defines wound-healing in a non-regenerating limb is the presence of fibrous connective tissue and of muscle elements, oriented perpendicularly to the ends of the cut bone-shafts. These elements have infiltrated distally to the vacuolated regions of the perichondrium and the hemopoietic area of the bone-marrow; they constitute a fibroid wall and in spite of the presence of numerous 'blastematous' cells further progress of regeneration is always prevented in this and in similar cases. As time progresses the stratified layers covering the skeletal structures will become, in cases of more advanced amputation age, much thicker and serve as an impenetrable callus over the cut bones. Another invariable concomitant of inhibited regeneration is the transformation of the periosteal tissue into massive cartilaginous formations.

Plate 2, fig. B is a section from the upper arm of a much younger frog. In contrast to the preceding case, this amputation surface completely lacks identifiable blastematous cells. Moreover, while showing the features of wound-healing enumerated above, this limb has a much thicker basement membrane and a more clearly defined cartilaginous cap immediately distal to the cut humerus at this later stage of cicatrization. It should be noted that this limb, whose humerus is made up exclusively of cartilage, and is therefore less structurally differentiated, demonstrates no greater capacity for dedifferentiation and regeneration than did the former limb.

Among the ten controls examined we have found no regeneration on gross inspection, and on slides we found no free, unencapsulated blastematous masses. Singer (1954) records regenerative response in 15 per cent. of the controls in 'recently metamorphosed frogs' while our own experiments, performed on much older frogs, corroborate the findings of Thornton & Shields (1945).

(*b*) *Amputations combined with interference with cicatrization processes* were performed in another series of fourteen *R. clamitans*, ten limbs of which were fixed 52 days after amputation and studied histologically.

Since early cicatrization is generally considered coincidental with non-regeneration it was thought advisable to test this proposition by means of surgical removal of the skin which, in frogs, precociously invades the amputation surfaces.

Plate 2, fig. C illustrates a case in which skin circumcision was performed twice, once on the seventh and once on the fourteenth day following amputation. The figure shows that normal wound-healing processes are operating, although they have been retarded. While the callus is well established over the skeletal shafts, and a connective tissue pad has formed distally, the basement membrane and skin glands have not yet been reconstituted over the whole amputation surface. The staining properties of some of the perichondrial elements visible

in this section suggests some dedifferentiation and blastema-like cell formations may be discerned anteriorly to the cut bone-shafts. This attempt at regeneration, however, has been halted by the fibrous layer capping them. The generality of this aspect of early regeneration 'interfered with' has been verified in the remaining nine cases of this series. The skin-removal treatment employed twice on each limb was therefore only able to retard wound-healing, not to induce a recovery of lost 'embryonic' properties of cells requisite for regeneration.

3. *Effects of adrenal transplants upon regeneration in hypophysectomized frogs* (25 postmetamorphic frogs—16 *R. clamitans* and 9 *R. pipiens*; mean body length, 4·4 cm., range, 4·1 to 4·9 cm.; 16 limbs investigated histologically)

The success in reawakening lost powers of regeneration in hypophysectomized newts by replacement therapy with cortical hormones (Schotté & Bierman, 1956) and with xenoplastic adrenal transplants (Schotté & Lindberg, 1954) suggested the use of a similar procedure in frogs. Concomitant with hypophysectomy one forelimb was amputated in all cases. The time and number of adrenal transplantations are summarized in Table 1 along with the data concerning the effects the transplants exerted on regeneration.

TABLE 1

Results of heteroplastic transplantations of adrenals after forelimb amputation in hypophysectomized frogs

(Groups marked with * were all *R. pipiens*, the other hosts were *R. clamitans*. All adrenals *R. pipiens*; one adrenal transplanted represents the adrenal tissue from one kidney)

		Number and order of transplantations of adrenals in respect to amp. age						*Macroscopic and histological results*				
		Day of administration						*Beginning of reg. observed*	*Gross regeneration*			
No. of frogs	*Level of amp.*	*I*	*II*	*III*	*IV*	*V*	*Total no. adrenals*	*No. days after amp.*	*Pres.*	*Abs.*	*Fixation after amp. (days)*	*Verified histo-logically*
9*	Distal humerus (8) Radius-ulna (1)	7	14	21	..	..	3	40–50	4	5	40–85	6
7	Distal humerus	3	3	3	10	10	5	40–50	3	4	75	3
9	Distal humerus	7	14	21	28	..	4	40–50	4	5	50	8

Macroscopic observation showed that during the 7 days preceding transplantation very rapid skin invasion occurred. Experiments to test the comparative rates of wound-healing in normal and hypophysectomized frogs confirmed that wound-healing was much more rapid in the latter: untreated hypophysectomized frogs at 15 days have a reformed dermis, epidermal skin glands, and a state of muscle-repair not yet present in limbs of normal frogs of the same amputational age.

In spite of rapid healing our data show that after adrenal transplantations, regeneration of some sort has occurred in eleven cases among the twenty-five examined. Excellent regeneration may be observed in the case represented on Plate 3, fig. D. After amputation through the radius-ulna, coincident with hypophysectomy, this frog received a total of three adrenal transplants, administered separately on the seventh, fourteenth, and twenty-first day following amputation. This case afforded the most extensive regeneration of the hypophysectomized hosts, and yet the amount of differentiation and of proliferation is less than in the majority of the normal hosts to be reported below. The section reproduced on the photomicrograph illustrates an abortive attempt at regenerative activity, since the most distal region of the limb remains blastematous and is not engulfed in connective tissue at this stage.

It should be made clear that the kind of regeneration observed in this series was different from that observed in normal hosts to be described below, for only the case just mentioned underwent proliferation, while the remaining limbs did not progress beyond the point of 'accumulation blastema' characteristic of the 'critical' phase of regeneration described by Forsyth (1946) for anuran premetamorphic tadpoles at non-regenerating levels. This is true even for the group of nine cases that received as much as five consecutive adrenal transplants without any significant increase in the frequency of regeneration (Table 1), which indicates that in hypophysectomized frogs the required adrenal threshold to restore regenerative capacity is much higher than in normal frogs. This result is in keeping with the general observation that in the absence of the pituitary cicatrization takes place much more rapidly, and it also suggests that in the absence of adrenocorticotropic stimulation the transplanted adrenals were at a low functional level.

4. *Effect of adrenal transplants upon regeneration in normal frogs* (16 postmetamorphic *R. clamitans*; mean body length, 4·9 cm., body range, 4·7 to 5·3 cm.; 15 limbs studied histologically)

Because previous investigations had shown extreme delays in blastema formation, the amputational surfaces in all cases but four were reopened by skin circumcision simultaneously with the introduction of the first transplant.

All the results from this series are summarized in Table 2. Among the twenty-one limbs (from sixteen individuals) studied only two presented no regeneration. When one considers that among the twenty-four individuals of the previously reported control group only two limbs exhibited any regeneration at all the importance of the adrenal factor introduced in these experiments appears impressive.

Our data show that the first signs of regeneration, in the form of macroscopically detectable blastematous mounds, appeared 30 days after the last transplant, at an amputation age of about 45 days (at 20° C.) in all but one exceptional case (see explanation in Table 2). The second observation which results from our

morphological and histological findings is that after an apparently normal start regeneration almost invariably becomes abnormal. A third general conclusion supporting the second centres around the disparity in stages of development at comparable amputation ages between anuran and urodele regeneration. This

TABLE 2

Results of heteroplastic transplantations of adrenals after forelimb amputation in normal R. clamitans

No. of frogs	Level of amp.	Day on which forelimb amp. performed: Left	Day on which forelimb amp. performed: Right	Day on which adrenal transpl. performed after amp. of right limb — Day of administration: I	II	III	Total no. adrenals	Macroscopic and histological results — Gross regeneration: Amp. age in days	Left: Pres.	Left: Abs.	Right: Pres.	Right: Abs.	Fixation after amp. days	Verified histologically
10	Radius-ulna	45 days later	1st day	7	14	..	2	45–50 (right) 85 (left)	1	0	10	0	65–160	9 (1)
4	Radius-ulna	1st day	33 days later	40	47	..	2	50 (left) 75 (right)	3	1*	4	0	75–160	5
2	Radius-ulna	..	1st day	7	14	21	3	45–50 (right)	..	..	1	1†	130	1 (1)

Special remarks: ( ) Individuals still alive and regenerating after 8 months (July 1957).
* The right limb of this case did regenerate.
† This right limb presents only instance of non-regeneration with amputation performed 7 days before transplantation.

is well illustrated by the figures of Plate 4, in which five morphologically successive normal stages and one abnormal regenerate are assembled. A regenerate 50 days old (Plate 4, fig. G) corresponds to a newt regenerate of about 20 days of amputation age; the regenerate 60 days old (Plate 4, fig. H) corresponds to a newt regenerate of about 25 days; the regenerate 72 days old (Plate 4, fig. I) is comparable to the 'palette' stage of a newt about 35 days old. Finally, Plate 4, fig. J, of an amputation age of 110 days is comparable to the regenerate of a newt at 40 days. This particular regenerate, FJW_{26}, was fixed, and a longitudinal section is represented on Plate 3, fig. E. Our records show that at the time of fixation this regenerate had not ceased to grow in length, an observation supported by the histological aspect of this section: while at its distal portion separate digital phalanges are discernible, the other bones of the forearm are still unindividualized.

The fifth normally regenerating case is represented by Plate 4, fig. K, and it shows clearly visible digital differentiations 130 days after amputation. A similar state of regeneration in an adult newt would have been achieved as early as 50 days after amputation. On slides this case showed clearly separated carpal, metacarpal, and phalangeal cartilaginous islets. However, a certain degree of syndactylism is evidenced by carpal fusion.

Frog regenerates of this type have the essential attributes of morphogenesis, growth, and differentiation that make these formations appear like true urodele regenerates. That this is not always the case has been observed in several instances where, instead of a regular terminal manus formation, a mushroom-like growth results. Such conditions may be discerned at the earliest beginnings of regenerative proliferation, and they are illustrated *in statu nascendi*, as it were, in Plate 3, fig. F. The lack of symmetry in cellular configuration is dramatized by the disorderly alignment of multiple cartilaginous and connective tissue whorls. It becomes understandable how after a long period (up to 8 months after amputation) continuous chaotic growth leads to a regenerate of abnormal appearance, such as has occurred in the left limb of case FJW_{19} (Plate 5, fig. M).

Not always, however, does abnormal regeneration of this type degenerate into a formation devoid of 'order'. In other cases, such as represented by Plate 4, fig. L, axial growth is at first normal, but the distal portions of the regenerate, no doubt due to early morphogenetic disorientation, are abnormal. On slides this case exhibits a normal stem formation, but where the manus should differentiate there is within a large cartilaginous terminal mass only a vague suggestion of subdivision, even as late as 7 months after amputation.

Such cases, where gross observation and histological verification show early differentiation of multiple cartilaginous cores, always degenerate into abnormal appendages, and they have been observed several times in this investigation. Similar results have also been obtained from another research performed at this laboratory following administrations of ACTH and of cortisone to amputated adult frogs (Schotté, unpublished).

That true regeneration, still not perfect but of a type not yet reported, may result from adrenal transplants is exemplified by the corresponding right limb of the same frog, FJW_{19} (Plate 5, figs. M and N), the photograph being taken after 8 months of regeneration. This regenerate has always kept the appearance of a normal blastema, and at the time of writing there is a somewhat syndactylous manus with, however, one individualized digit and another finger (as indicated on the X-ray picture) with separated metacarpals and at least two phalanges fused with the rest of the manus.

To recapitulate the data from this series of experiments, it may be stated that the addition of the equivalent of the adrenal complement from one adult frog (administered within one to two weeks after amputation) has brought about regeneration in all but one post-metamorphic frog. The most surprising new finding was that in three cases the delayed transplantation of adrenals brought about regeneration in limbs which had visibly healed over their amputational surfaces.

DISCUSSION

This research has been undertaken to investigate whether or not the lost regenerative capacity in adult frogs could be reinstated by introducing additional

adrenal glands. The results provide an affirmative answer to this question only so far as the positive action of the adrenals is concerned, for an investigation of the separate effects of other tissues, glands, or substances is not yet concluded. We are therefore unable at this time to attribute the restoration of regenerative capacity in frogs to the direct and exclusive action of the transplanted frog adrenals. In this respect it is imperative to test the possible role of kidney tissue, as it was impossible to entirely separate kidney elements from the adrenal islets by ordinary surgical procedures. Moreover, preliminary experiments performed with newts have revealed a complex situation in respect to the possible stressor effect of actively secreting and therefore poisonous and irritating transplanted kidney tissues. Since stress in Selye's sense invariably involves the pituitary-adrenal axis, it would not be surprising to discover that transplanted kidneys also are capable of creating a hyperadrenal state conducive to regeneration (Schotté & Lindberg, 1954).

Before concluding that the adrenal transplants are the sole agents in bringing about regeneration in adult frogs, the importance of skin removal and of amputational stress must first be considered because of the possible stressor role of surgical trauma in newts (Pellman & Schotté, 1955; Lindberg & Schotté, 1955; Schotté & Bonneville, 1955). The amputation alone of another limb must be excluded as a stressor agent capable of promoting regeneration, since it was followed in all cases simply by the normal wound-healing mechanism. Secondly, concerning the effects of skin circumcision, only one of the fourteen forelimbs upon which repeated skin removal around the amputational surface was performed exhibited any regeneration, and such a regenerate did not continue growth beyond the accumulation phase. Thus, this treatment did not restore any regenerative activity comparable to that observed in the transplantation experiments.

These brief considerations will suffice to re-emphasize the belief that the results obtained are attributable to the introduction of additional adrenals. But then to what effect of the transplanted adrenals may one attribute the induced regeneration?

Since experimentation in this laboratory has implicated a pituitary-adrenal synergism in establishing conditions favourable to regeneration in newts, one wonders whether such a mechanism is operative in adult frogs also. Unpublished investigations regarding the effects of stress upon adult frogs and adrenal transplants upon regeneration in tadpoles would intimate that this is so. However, to support this hypothesis, an important piece of information is required. Were the adrenal transplants synthesizing cortico-steroids found to be essential for the initiation of regeneration in urodeles (Schotté & Bierman, 1956)? Besides the gross and microscopical condition of the adrenal tissues described in the experimental part there is additional evidence to indicate that the adrenal transplants were functioning, but at a basal level.

Firstly, in hypophysectomized frogs the bright yellow pigmentation of the

adrenals faded in a manner identical to that in normal hosts receiving high adrenal dosages by transplantation. The paleness of the hypophysectomized host's own adrenals was no doubt due to the fact that involution of the adrenal cortex, in the absence of ACTH, produces a lesser concentration of the yellow-coloured lipids in the cortical cells. The atrophy in the normal hosts receiving additional adrenals implies that the transplanted adrenals were functional, since in the endocrine system compensation for more than normal concentrations of a hormone is by atrophy of the organ synthesizing it. Secondly, bioassays of cortical secretions from isolated mammalian adrenal glands through which blood was perfused showed that deprivation of ACTH did not interrupt, but only lowered their secretory activity (Vogt, 1951). This result suggests that the frog cortices would not completely terminate their activity in the absence of the trophic hormone.

The next important problem concerns the duration of activity of the transplants. Both these results and those from a previous study in newts (Schotté & Lindberg, 1954) imply that there is a transitory period of corticoid activity, for in both experiments regeneration was only associated with transplantations made around two weeks after amputation, not when adrenals were introduced earlier. Apparently the period of cortical activity must be integrated with the time, late in wound repair, that is most susceptible to corticoid action. The failure to induce regeneration with smaller dosages in other experiments was probably not the result of inadequate quantitative dosage, but the result of premature administration, before the limb tissues were sensitive to proper interaction with cortico-steroids like cortisone. For this hormone is known to effect many changes in wound repair, such as diminishing cellular migration and infiltration, inhibiting fibroblast formation, and fibrin deposition (Cameron, 1953); and all these responses repress normal wound-healing processes which in turn interfere with the mechanisms of regeneration.

If these propositions regarding the delicate hormonal changes at the propitious moments when the frogs' limbs are in a state of repair most receptive to their action are valid, why is it that the adrenal transplants are unable to facilitate regeneration in hypophysectomized frogs to the same degree? It seems that there may be two main reasons. Firstly, the total absence of ACTH in animals deprived of their pituitaries is no doubt responsible for a much lower level of activity of the transplanted adrenal glands in these frogs; in normal hosts, however, after vascular communication has been established, ACTH is present for cortical activation. Secondly, the much higher rate of cicatrization observed in hypophysectomized frogs possibly modifies the time at which the corticoid activity is most instrumental in preventing wound-healing.

The general results of this study, then, together with the still unpublished aforementioned experimental findings, support the proposition that a pituitary-adrenal synergism is operating in adult frogs as in newts; they also suggest that an artificially-induced hyperadrenal state is sufficient to determine recuperation

of regenerative capacities in adult frogs. This, of course, does not preclude other factors (nerves, for example) from being just as effective in restoring regenerative potencies.

In conclusion it may be stated with a certain degree of confidence that the normal loss of regenerative potencies in Anura, more or less coincidental with metamorphosis, is attributable to endocrine changes in these organisms, rather than to irreversible modifications in properties of the cellular constituents of their limbs.

SUMMARY

1. Experiments to test the proposition that systemic factors and not fixed properties of the cells within limbs determine loss of regeneration in frogs were performed upon the forelimbs of post-metamorphic *R. clamitans* and *R. pipiens* varying in body length from 4·4 to 5·7 cm.

2. Control experiments consisting of simple amputations through the mid-radius ulnar or distal humeral regions showed that the frogs at this stage were incapable of detectable regeneration. The repeated removal of the cicatricial skin in another control series elicited regeneration, of an abortive type, in only one case among fourteen limbs tested.

3. In twenty-five hypophysectomized *R. clamitans* adrenals from *R. pipiens* were transplanted heterotopically beneath the jaw concomitantly with amputation. Weak regenerative response was observed in eleven cases. It is concluded that the absence of the pituitary in the hosts lowers the activity of the transplanted adrenals.

4. The transplantation of *R. pipiens* adrenals into normal *R. clamitans* hosts 7 and 14 days after amputation proved most effective, since among the twenty-one limbs examined only two did not show any regeneration.

5. It is suggested that: (1) the transplanted adrenals were functional, although at a low level of activity; (2) the time of transplantation of the adrenals is critical, since in order to elicit regeneration it must coincide with a period in amputational wound repair that is most susceptible to corticoid activity; (3) the loss of regenerative potencies after metamorphosis in Anura is attributable to changes in the endocrine system of these organisms, rather than to changes in the properties of the cells of their limbs.

ACKNOWLEDGEMENTS

Thanks are due to the Division of Grants of the U.S. National Institutes of Health (Grant 2236) which has made this research possible. We also wish to thank Mrs. Jean Francis for excellent histological work and Mr. Carl Howard for consummate skill in photography.

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EXPLANATION OF PLATES

PLATE 1

Ventral view of lower jaw of an adult *R. clamitans* (case FJW_9) after removal of skin showing four prominent adrenal transplants (from *R. pipiens* donors) 125 or 118 days after transplantation. Several smaller islets below derive from other unsuccessful or regressing adrenal transplants. Note vascular connexions of the four main transplants.

PLATE 2

FIG. A. Photomicrograph of a longitudinal section of left forelimb of case FJW_{67} amputated through the radius-ulnar region and fixed after 23 days of amputation age. Note reformation of skin over amputational surface, complete with basal membrane. Also characteristic for arrested regeneration are the masses of procartilage formed on both sides of the periosteum at the expense of periosteal and other connective tissue cells. (×25)

FIG. B. Photomicrograph of a longitudinal section of left forelimb of case AFC_{82}, amputated through distal humerus and fixed 50 days after amputation. Note lack of blastematous cells and the cartilaginous nature of the humerus. (×25)

FIG. C. Photomicrograph of a longitudinal section of right forelimb of case FJW_{93} amputated through the radius-ulnar level and fixed 50 days later. Note the incompletely reformed basement membrane, the crescent-like pad of fibrous tissue distal to the cut shafts of the bone collar, and the dense periosteal cartilage. (×25)

PLATE 3

FIG. D. Photomicrograph of the forelimb of case FJW_{24} fixed 120 days after amputation. The amputation level is situated at the bottom of the figure where the old bony formations are still visible. Within the regenerated procartilaginous mass forming the distal portion of the radius and the general, still not separated mass of the manus, may be distinguished small hemopoietic islets. From the aspect of the distal skeletal formations it is doubtful whether digital formations would ever emerge. (×25)

FIG. E. FJW_{26}. Photomicrograph of a longitudinal section of left forelimb of case FJW_{26}, at 110 days amputation age. Radius and ulna are well regenerated and elements of the basipodium of the metacarpals and even the phalanges are well indicated on this and on other sections. (×25)

FIG. F. Photomicrograph of a longitudinal section of the right forelimb of case FJW_9 fixed 60 days after amputation and 46 days after the second adrenal transplantation. The long cartilaginous shaft is the radius and ulna, probably fused, around which periosteum begins to form. From the distal area of these procartilaginous formations several whorls of cartilage are derived which have distinct and irregularly distributed centres. Since there is no regular connective tissue cap precluding further growth, these irregular blastematous centres may diverge into tridimensional, often digit-like formations which eventually form a mushroom-shaped regenerate. (×25)

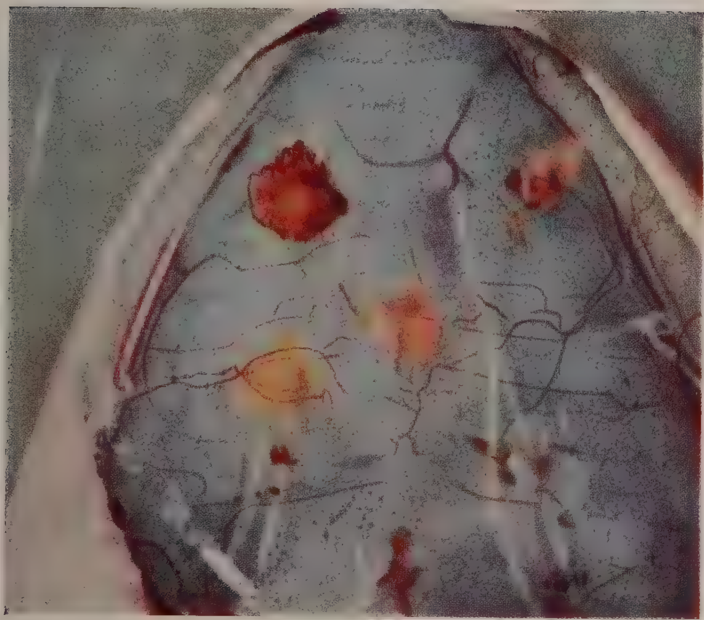
PLATE 4

All figures here represented are regenerates from hosts which received two successive *R. pipiens* adrenal transplants 7 and 14 days after amputation of the forelimb.

FIG. G. *R. clamitans* forelimb regenerate at the stage of early blastema, 50 days after amputation.

FIG. H. *R. clamitans* regenerate at the blastema stage, 60 days after amputation.

FIG. I. *R. clamitans* regenerate at the stage of flattened palette, 72 days after amputation.



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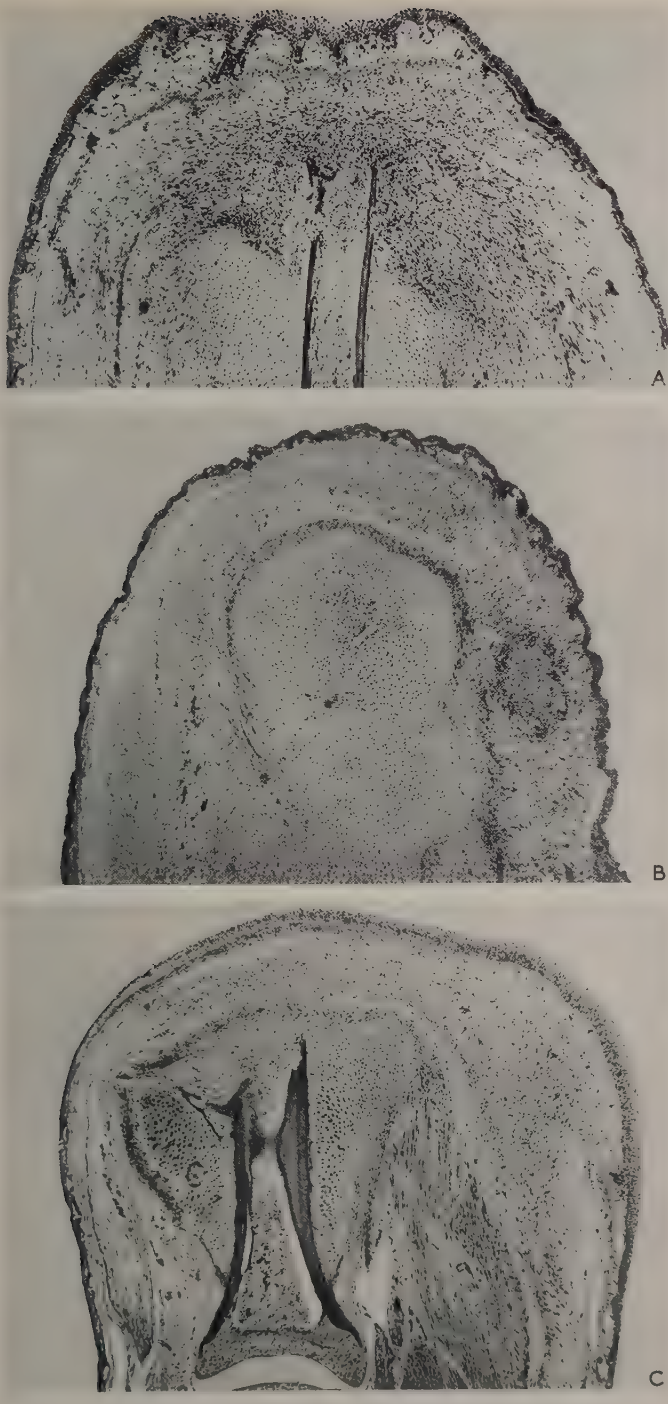

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Plate 2

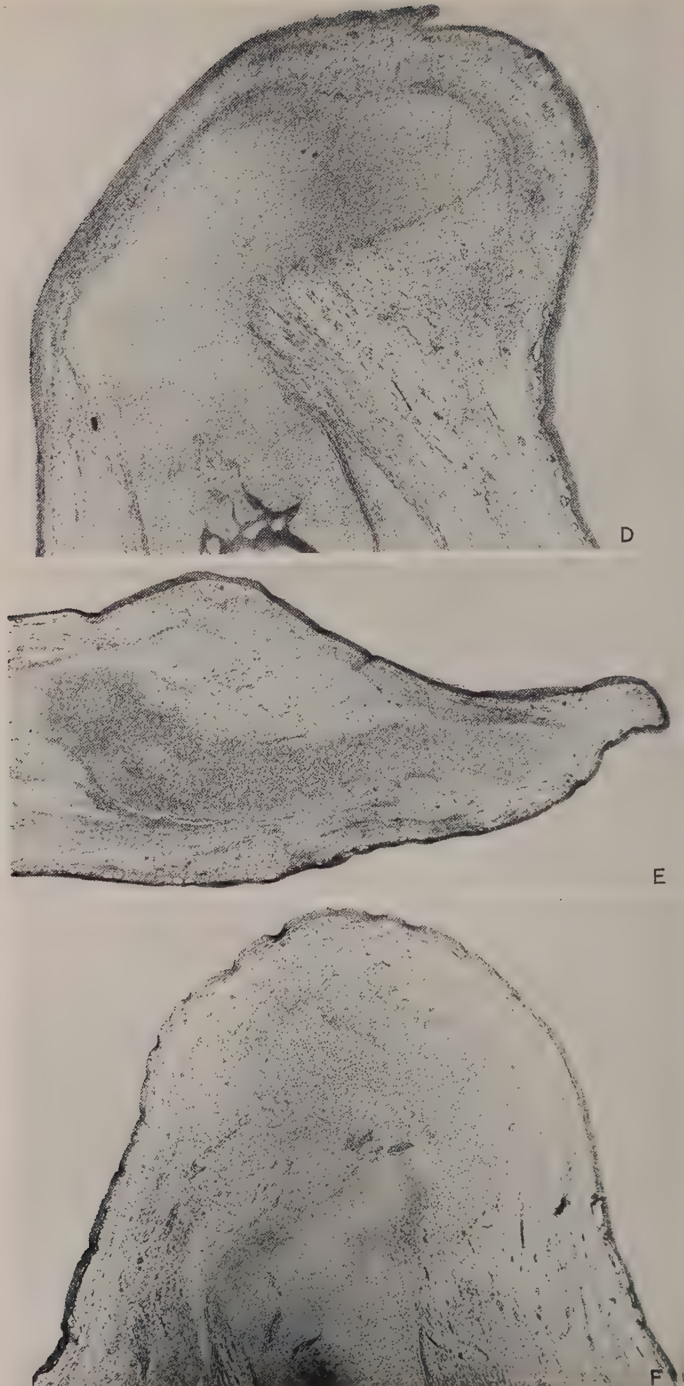

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Plate 3

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Plate 4


M


N

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Plate 5

FIG. J. *R. clamitans* forelimb regenerate 110 days after amputation. (A photomicrograph of a section of that limb is reproduced on Plate 3, fig. E.)

FIG. K. Forelimb regenerate at the stage of prominent digital differentiation, 130 days after amputation, showing clearly defined three digital indentations.

FIG. L. Abnormal regenerate from forelimb amputated through the proximal radius-ulna showing a boxing-glove-like curvature at its tip, 7 months after amputation.

PLATE 5

FIG. M. Ventral view of *R. clamitans*, 5·3 cm. body length, and amputated bilaterally, the left limb, however, amputated 45 days after the right (case FJW_{19}). Two adrenal transplantations were performed 7 and 14 days respectively after amputation of the right limb. Note mushroom-like regenerate at left and excellent regeneration with digital differentiations at right.

FIG. N. X-ray photograph of the right limb of the above case taken 8 months after amputation and adrenal transplantations. Note the fused osseus rod of radius-ulna, some carpal formations and particularly phalangeal ossifications within the free digit.

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Effect of a Leucine Analogue on Incorporation of Glycine into the Proteins of Explanted Chick Embryos

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AMINO acid analogues have been observed to give rise to abnormal forms of development of chick and amphibian embryos (Herrmann, 1953; Rothfels, 1954; Waddington & Sirlin, 1954; Feldman & Waddington, 1955; Herrmann, Rothfels-Konigsberg, & Curry, 1955). Assuming that these disturbances may be due to interference with the utilization of amino acids for protein formation, we have attempted an analysis of this effect by comparison of the protein contents and of the uptake of glycine into the proteins of chick embryo explants in the presence and absence of amino acid analogues. The results of such experiments are reported in this paper.

MATERIALS AND METHODS

The chick embryos used for explantation, the explantation technique, and the determination of total protein glycine and of tracer glycine were essentially the same as described previously (Herrmann & Schultz, 1958). The embryos were explanted at the 11–13 somite stage on to the surface of an agar gel containing egg extract as nutrient medium following the procedure given by Spratt (1947) as modified by Rothfels (1954). For the measurement of tracer uptake 20–25 μg. of glycine-1-C^{14} with a radioactivity of 1 μc. was added to the warm, liquid agar-medium solution and plated out with this mixture. The amino acid analogue used in the present experiments was ω-bromoallylglycine (BAG) which is regarded as an analogue of the amino acids leucine and valine. It was added to the medium in the same way as the tracer in a final concentration of 0·15 mg./ml.

Previous experiments (Herrmann & Schultz, 1958) have shown that the uptake of labelled glycine deviates only slightly from a straight line course for the first 4 hours of explantation. Therefore an incubation time of 4 hours was chosen as optimal incubation period throughout the present experiments. After incubation the embryos were collected, washed for 10 seconds in each of three separate por-

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tions of 100 ml. saline, the extraembryonic membranes were carefully removed and individual organ primordia were dissected from the embryos and fixed in 5 per cent. trichloracetic acid, under the dissecting microscope. Nucleic acids and lipoids were removed by extraction with hot trichloracetic acid and with alcohol-ether followed by alcohol-chloroform respectively. Possibly adhering tracer glycine was removed by washing with a 1 per cent. glycine solution followed by six washings with distilled water. The proteins were hydrolysed and the protein glycine was isolated chromatographically as dinitrofluorobenzene derivative (Krol, 1952). The quantity of the intensely yellow glycine derivative was determined spectrophotometrically and the radioactivity of the isolated samples was measured by counting the glycine compound as an infinitely thin layer in a gas-flow counter with about 20 per cent. efficiency. The ratio of counts/min./μg. glycine in the tracer glycine was used as conversion factor for calculation of microgrammes of tracer glycine in the samples. Specific activities are given as the ratio of tracer glycine/total glycine.

Calculations of the means and standard errors of specific activities, tracer protein glycine and total protein glycine were carried out in the conventional way. The differences between specific activities of the organ primordia listed in Table 1 and between the contents of tracer protein glycine or total protein glycine, obtained for one and the same set of embryos in the presence or absence of the analogue, have been calculated for each separate experiment. The means and standard errors for these 'paired' difference values were used for evaluation of their statistical significance.

RESULTS

From the data compiled in Table 1 it is apparent that the uptake of tracer glycine into the proteins of the tissues of the embryo explants differs widely. In terms of specific activities the highest values were found in the proteins of the primitive knot, followed closely by the values obtained for the spinal cord, with somewhat lower values in the somites and the segmental plate,[1] and the lowest figures for the brain, heart, and notochord. With the number of determinations carried out so far no statistically significant differences could be established between the specific activities of the primitive knot, the spinal cord, and the somites. The specific activities of these tissues are, however, significantly higher than those of the brain, the heart, and the notochord. Of these latter tissues the brain is significantly higher in activity than the heart, and the heart, in turn, has a significantly higher activity than the notochord. One can see that in one case morphogenetically closely related tissues, like segmental plate and somites, have an uptake of the same order of magnitude although their state of differentiation has to be assumed to differ considerably. On the other hand, brain and spinal cord, tissues of a similar morphogenetic relationship, show widely differing incorporation rates.

[1] The axial mesoderm caudal to its segmented portion as defined by Spratt (1955).

TABLE 1

Uptake of tracer glycine into proteins of primordia of explanted chick embryos

(Specific activities given as microgrammes protein glycine-1-C^{14}/total protein glycine $\times 10^3$ (mean $\pm$ S.E.). The data listed in III to VIII give the differences (mean $\pm$ S.E.) between specific activities of primordia listed at the head of III to VIII and in I respectively)

I	*Specific activities* II	*Neural tube* III	*Somites* IV	*Segmental plate* V	*Brain* VI	*Heart* VII	*Notochord* VIII
Primitive knot 6* 10†	7·02±0·90	1·11±1·47 $P > 0·4$ $< 0·5$	1·28±1·00 $P > 0·2$ $< 0·3$	2·92±0·84 $P > 0·01$ $< 0·02$	3·10±1·15 $P > 0·02$ $< 0·05$	4·94±0·74 $P > 0·001$ $< 0·01$	5·82±0·83 $P < 0·001$
Neural tube 9* 10†	6·20±1·26		1·21±0·79 $P > 0·1$ $< 0·2$	2·06±1·02 $P > 0·05$ $< 0·1$	2·58±0·89 $P > 0·01$ $< 0·02$	4·23±1·20 $P > 0·001$ $< 0·01$	5·02±1·12 $P > 0·001$ $< 0·01$
Somites 9* 200–50†	4·99±0·82			0·85±0·72 $P > 0·2$ $< 0·3$	1·37±0·49 $P > 0·02$ $< 0·05$	3·02±0·70 $P > 0·001$ $< 0·01$	3·80±0·71 $P < 0·001$
Segmental plate 9* 10†	4·14±0·55				0·52±0·59 $P > 0·4$ $< 0·5$	2·17±0·39 $P < 0·001$	2·96±0·41 $P < 0·001$
Brain 9* 10†	3·62±0·50					1·65±0·45 $P > 0·001$ $< 0·01$	2·44±0·42 $P < 0·001$
Heart 9* 10†	1·97±0·22						0·79±0·13 $P < 0·001$
Notochord 9* 10†	1·18±0·18						

* Number of determinations.
† Number of primordia used for each determination.

TABLE 2

Comparison of protein glycine content of the whole embryo determined directly with that derived from measurements of individual primordia

Organ	*Microgrammes total protein glycine in single organ*		*Microgrammes protein glycine-1-C^{14} $\times 10^3$*	
	Controls	*Experimental*	*Controls*	*Experimental*
Primitive knot	0·35	0·28	2·34	1·80
Neural tube	0·45	0·42	2·55	2·54
Somites	0·60	0·48	3·00	3·28
Segmental plate	0·36	0·30	1·24	1·28
Brain	0·53	0·51	1·91	1·98
Heart	0·38	0·33	0·74	0·87
Notochord	0·32	0·23	0·33	0·32
TOTAL	2·99	2·55	12·11	12·07
Determination on whole single embryo	3·32	..	42·73	..
Per cent. primordia/whole embryo	90	..	28	..

From the data recorded in Table 2, it can be seen that the sum of the total protein glycine values of the dissected primordia is only 10 per cent. lower than the corresponding value of the embryo as a whole. In contrast, the sum of the values for the protein glycine-1-C^{14} of the separate primordia is only about 30 per cent. of the tracer content of the whole embryo. This must mean that a tissue which accounts for less than 10 per cent. of the protein content contains about 70 per cent. of tracer glycine of the whole embryo. Since the endoderm is not included among the dissected primordia, and since it is the tissue in direct contact with the tracer-containing medium, it is considered as the possible site of such a high tracer incorporation.

The wide range of incorporation rates found in the organs of explanted chick embryos by our quantitative measurements was not observed in radioautographic estimations by Feldman & Waddington (1955) obtained in chick embryos *in vivo*. This absence of larger differences could be due to a greater

TABLE 3

Contents of tracer protein glycine and total protein glycine in primordia of chick embryos explanted for four hours in the absence and presence of ω-bromoallylglycine (0·15 *mg./ml.*)

(Number of determinations and of primordia used as in Table 1)

	Microgrammes glycine-1-$C^{14} \times 10^3$			*Microgrammes total protein glycine*		
	Control Mean±S.E.	*Analogue Mean±S.E.*	*Difference Mean±S.E.*	*Control Mean±S.E.*	*Analogue Mean±S.E.*	*Difference Mean±S.E.*
Primitive knot	23·36±3·27	17·99±1·74	5·38±1·95 $P > 0·02$, $< 0·05$	3·45±0·46	2·80±0·36	0·81±0·36 $P > 0·05$
Neural tube .	25·45±3·85	25·44±2·34	0·003±2·89 $P > 1·00$	4·53±0·52	4·18±0·37	0·042±0·56 $P > 1·00$
Somites .	20·59±2·95	21·84±3·18	1·24±1·87 $P > 0·5$	4·23±0·41	3·20±0·24	1·03±0·25 $P > 0·001$, $< 0·01$
Segmental plate	12·33±1·14	12·76±1·22	0·48±0·89 $P > 0·6$	3·62±0·87	3·03±0·40	0·56±0·59 $P > 0·3$
Brain . .	19·14±2·20	19·85±2·14	1·71±1·29 $P > 0·2$	5·29±0·61	5·12±0·44	0·18±0·65 $P > 0·7$
Heart . .	7·38±0·95	8·67±1·20	1·53±0·93 $P > 0·1$	3·83±0·44	3·31±0·55	0·52±0·58 $P > 0·3$
Notochord .	3·32±0·35	3·21±0·19	0·11±0·24 $P > 0·6$	3·20±0·57	2·34±0·28	0·86±0·53 $P > 0·1$

uniformity of the considerably higher growth rates of the tissues when developing in the egg as compared to the residual growth prevailing during cultivation *in vitro* under conditions of the present experiments. However, the long incubation in the presence of tracer glycine for periods of 32–42 hours as used in the experiments of Feldman & Waddington (1955) may eliminate differences observable after shorter incubations. In particular it has to be considered that under Feldman & Waddington's conditions, and in the absence of time curves for

tracer incorporation, some tissues may still show an increasing tracer accumulation while in others the tracer content may already be declining. Thus apparently similar tracer values may represent entirely different stages of incorporation. It should also be pointed out that radioautographic analyses do not lend themselves readily to the establishment of determinations of specific activities. A higher grain count may represent, at least in part, a difference in the protein concentration in the observed tissues. Such considerations may explain the discrepancy between the large differences of incorporation between notochord and neural tube found in our experiments as compared to the apparently similar incorporations for the same tissues apparent in the radioautographs of Pantelouris & Mulherkar (1957).

On addition of the bromoallylglycine to the medium statistically significant changes in the measured parameters were found in the case of the somites and of the primitive knot (Table 3). In the former the total protein glycine decreased on cultivation of the embryos in the presence of the analogue while the tracer glycine content remained apparently unchanged. In the case of the primitive knot tissue, both the total protein glycine and the tracer glycine show a significant decrease. No changes of statistically unequivocal significance could be observed in the other organ primordia.

DISCUSSION

It was pointed out in another paper (Herrmann & Schultz, 1958) that compared with embryos developing in the egg the growth rate of the explanted embryos is low. On the other hand, differentiation as measured, for example, by the formation of somites, of the heart tube, or of the brain ventricles and eye stalks, seems to proceed at a practically undiminished rate. At the present time it is not possible to state whether the observed differential rates of incorporation of tracer amino acids into the proteins of the different organ primordia are connected with the residual growth or with the formation of new proteins during differentiation. If related to residual growth, incorporation rates and growth rates (protein accumulation) in the different primordia should run parallel. In explants, however, growth measured in terms of protein accumulation is so slow that differences in the growth rates of the primordia could not be established under the conditions of the present experiments. Even measurements of growth rates of individual organs at early stages of chick embryogenesis *in vivo* are as yet too incomplete to allow a closer comparison of incorporation rates in explants and the actual growth rates observed in the embryo developing in the egg. From data obtained by Schmalhausen (1926) it would seem that the fresh weight of the heart and of the brain increases about 4–5 times from the third to the fourth day of incubation. In comparison, the protein nitrogen of an average somite (Herrmann *et al.*, 1951) increases by about the same ratio during the development from the second to the third day and a doubling of the somite proteins was found during the third to fourth day period. Tracer incorporation,

however, was found to be significantly higher in the somite proteins than in the protein moiety of the heart and the brain. If Schmalhausen's (1926) measurements can be substantiated, the growth rates, at least when observed *in vivo*, cannot be related to incorporation rates found in explants.

In this context it should be mentioned that according to Schmalhausen (1926) growth and differentiation during early stages of development occur in alternating spurts of abruptly increasing or decreasing rates. For the comparison of incorporation rates of different organ primordia assessment of their state with respect to these developmental phases may be desirable. Further investigation of the relations of incorporation and protein accumulation (growth) and differentiation will be made by attempting to vary the growth rates of the explants and by comparison of incorporation rates and protein accumulation under these conditions.

It is noteworthy that bromoallylglycine at the concentration used in the present experiments (about 0·001 M) affects the measured parameters only in two primordia, the somites and the primitive knot. In both instances addition of the analogue to the medium leads to a lower total protein glycine content. Since no significant net protein increase could be detected in whole explanted embryos, the assumption of an inhibition of protein accumulation would seem less likely than the assumption of a protein deficit due to increased protein degradation. This can lead apparently to a lowering of both the tracer glycine and the total protein glycine as in the case of the primitive knot. In the somites, loss of total protein glycine occurs while the content of tracer glycine is practically the same in explants cultivated with or without analogues. In this case one can postulate that the loss in total protein content is partially compensated by newly formed proteins containing tracer. As an alternative interpretation one can assume that the proteins in the somites may have a finite life span, like haemoglobin (Shemin & Rittenberg, 1946) or muscle proteins (Dreyfus *et al.*, 1956). In this case protein loss could occur by degradation of the 'older' unlabelled proteins without decrease of the tracer content confined to the 'younger' proteins, which would remain undegraded during the experimental explantation period.

If these conclusions are correct, the increased protein degradation found in the presence of bromoallylglycine would corroborate observations by Jensen, Lehmann, & Weber (1956), who found an increased catheptic activity in amphibian tail tissue after administration of leucine analogues. To what extent the protein loss in the present experiments can be attributed to an increased proteolysis by cathepsin will be determined in future experiments. It should be pointed out that *o*-fluorophenylalanine, an analogue of phenylalanine, was found to be without effect on the catheptic activity of liver homogenates. In liver slices, it inhibited both the protein synthesis and protein degradation (Steinberg & Vaughan, 1956). However, bromoallylglycine gives rise to far-reaching degenerative changes in the cells of the somites which may be related to the more

extensive protein degradation discussed above. The correlation of protein loss and the degenerative processes in the somites will be discussed in a separate paper (P. W. Schultz, to be published).

SUMMARY

1. The incorporation of glycine-l-C^{14} into the chromatographically isolated protein glycine of the primordia of explanted chick embryos was determined quantitatively.

2. According to decreasing tracer uptake, the following order of the tested primordia was found: primitive knot, spinal cord, somites, segmental plate, brain, heart, and notochord.

3. Presence of ω-bromoallylglycine in the medium (0·15 mg./ml.) leads to a lowered total protein content in the somites and in the primitive knot and a lowered tracer glycine content in the latter.

4. The possible relation of these results to the development of the organ primordia and their protein metabolism is discussed.

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Rates of Growth and of Remodelling as Factors in the Genesis of Vascular and Osseous Lesions of Odoratism in Rats

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WITH ONE PLATE

INTRODUCTION

SINCE the original report by Geiger, Steenbock, & Parsons (1933), the syndrome of experimental odoratism, including aortic aneurysms and ruptures, skeletal deformities and herniae, has been reported as reproducible with considerable consistency in weanling rats fed either the seeds of the sweet pea (*Lathyrus odoratus*) or the extractable active nitriles (Strong, 1956). The vascular lesions have recently received special attention by a number of investigators (Bachuber & Lalich, 1955; Churchill *et al.*, 1955; Walker & Wirtschafter, 1956; Menzies & Mills, 1957), the general consensus of present opinion being that the dramatic aortic aneurysms and ruptures are primarily attributable to 'elastinolysis'. While Walker & Wirtschafter (1956) considered the aortic lesions to be '. . . one sign of a generalised metabolic disorder which at the histological level is basically elastolytic', neither they nor other workers, to our knowledge, have attempted to interpet, within a *single* framework *all* the above-mentioned lesions of odoratism, nor have attempts yet been made to explain particularly the marked variations in susceptibility to the odoratus toxin of weanlings as compared with adult rats.

Yet several studies have already shown that the lesions in odoratism seem somehow to be bound up with age or the stage of development of affected animals (Ponseti & Shepard, 1954; Chang *et al.*, 1955) and even that connective tissues may be diffusely involved throughout the organism (Dasler, 1954). However, the reasons underlying these influences of age and/or of developmental activity on the susceptibility of individuals to the toxic effects of odoratus seeds or to the extractable toxic nitriles have not been discussed at any length by others.

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Our own orientation to this problem was initially from the standpoint of collagen synthesis and related biochemical changes during wound healing, although our initial histological studies of healing wounds in odoratus-intoxicated rats have not yet revealed easily detectable differences from the normal. However, we anticipate that the quantitative chemical analyses of this problem, presently being undertaken, may perhaps be more revealing. We had, nevertheless, already disclosed indications that the synthesis of elastic tissue and of collagen were intimately related with ground substance metabolism in maintaining the integrity of healthy vascular elastic membranes (VEM's) (Gillman, Hathorn, & Penn, 1957). We have also found that, following injury, the regeneration and/or repair of VEM's is closely comparable in many respects with the serial histological and histochemical events observable in the healing of cutaneous wounds. The massive accumulations of metachromatic and ferrocyanide-positive polysaccharides in the aortic wall, detectable even within 14 days of initiating odoratus intoxication, led us to consider that it was the remodelling and associated lysis and reconstruction of VEM's and of long bones, during periods of active growth, which made young animals so particularly susceptible to the toxic effects of sweet-pea seeds. This encouraged us to re-examine the literature as well as to undertake further original studies both of odoratism and of the post-natal growth of normal rats. Our initial findings, relating only to some aspects of the aortic and osseous lesions, here reported in outline, are believed to lend some support to this basis for accounting for both the vascular and the osseous lesions in odoratus intoxication.

If these views can be substantiated by quantitative radio-active tracer studies, then a new orientation may be provided for further analyses of several types of vascular and osseous lesions and of some forms of vascular sclerosis and especially for the 'pseudo-elastic tissue' accumulations associated with ageing or following chronic injury in man (Gillman *et al.*, 1955; Gillman, Hathorn, & Penn, 1957). Even more important, perhaps, is the possibility that lesions, hitherto regarded as peculiar to the arteries of ageing individuals, may yet be shown to be simply the late sequelae of disturbances in the growth and remodelling of the vascular tree, especially during that period of active growth which characterizes adolescence (Tanner, 1955).

Such possibilities may also underline the need previously stressed (Gillman, Hathorn, & Penn, 1957; Gillman & Hathorn, 1957), for regarding sclerosis and other alterations in many connective tissues, however specialized they may at first seem to be, in terms of modern knowledge of the changes in and the factors regulating connective tissue regeneration or repair which have recently been disclosed by studies of healing cutaneous injuries (Kodicek & Loewi, 1955; Abercrombie *et al.*, 1954, 1956, 1957; Dunphy *et al.*, 1955, 1956; Udupa *et al.*, 1956; Gillman & Penn, 1957).

MATERIALS AND METHODS

In our studies of odoratism we have fed both weanling and adult rats with sweet-pea seeds. As originally shown by Geiger *et al*. (1933), and later by Ponseti & Shepard (1954), adults are also susceptible to the toxic actions of the active principle, albeit less so than weanlings and in less dramatic ways.

In our first experiment, 10 male weanling Wistar-strain rats aged 23–25 days (mean initial weight 38 g.) and 10 male adult rats aged about $2\frac{1}{2}$ months (mean initial weight 211 g.) were fed our stock diet with the addition of 50 per cent. ground sweet-pea seeds. Equal numbers of weanling and adult male control rats were fed the stock diet alone. Two experimental and 2 control rats, in both the weanling and adult groups, were sacrificed by ether anaesthesia at approximately fortnightly intervals, with the exception of 2 weanling rats on the sweet-pea seed diet (out of 4 remaining at the time) which died spontaneously of aortic ruptures at 52 and 53 days respectively.

In the second experiment, 10 male weanling rats aged 26–27 days (mean initial weight 48 g.) and 10 male adult rats aged about 3 months (mean initial weight 235 g.) were fed the above sweet-pea seed diet, an equal number of control rats being simultaneously fed the stock diet. In this experiment, however, the animals were sacrificed only when moribund or paralysed or at the end of the experiment lasting 216 days.

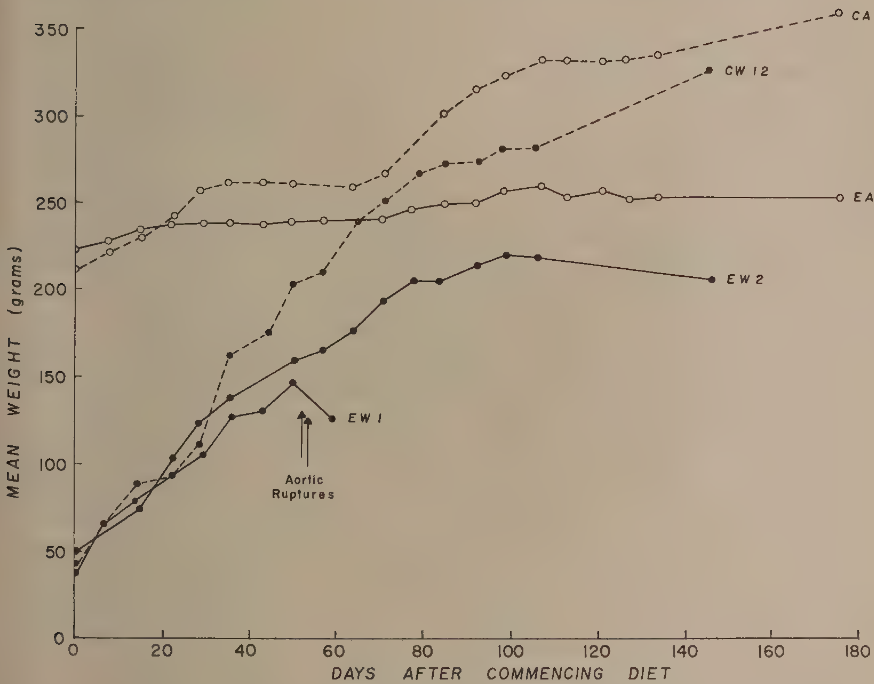
Immediately after sacrifice and macroscopic post-morten examination, the animals were fixed *in toto* in 10 per cent. neutral formalin and, 1 week later, specimens of heart, aortic arch, thoracic and abdominal aortae, humerus, elbow-, wrist-, and knee-joints, and the upper end of the femora were taken for decalcification (in the case of bones), dehydration, and subsequent paraffin embedding and sectioning. As described in other studies (e.g. Gillman, Hathorn, & Penn, 1957) *serial* sections of all specimens were stained with haematoxylin and eosin, a modified Masson method, the periodic-acid Schiff (PAS) routine, alcoholic toluidine blue, a ferrocyanide method for mucopolysaccharides (Rinehart & Abul-Haj, 1951), and the Wilder reticulin method counterstained with Van Gieson.

OBSERVATIONS

General

Text-fig. 1 illustrates the weight changes occurring in each group of animals. It will be seen that the weanling rats on the *L. odoratus* diet continued to grow, albeit at a slower rate than the weanling controls, until about 100 days, when their weight curve tended to plateau. The aortic ruptures that occurred at 52 and 53 days (in 2 of the 4 rats still surviving at this stage of the first experiment) nevertheless took place almost immediately after a period of rapid growth. The other interesting fact illustrated by Text-fig. 1 is that, over the period of the

experiment, the rats which were started on the odoratus diet when they were already adults grew hardly at all as compared with the adult controls.



TEXT-FIG. 1. Mean weight changes of experimental and control rats. EW 1: experiment 1; weanlings initially 23–25 days old fed 50 per cent. *L. odoratus* seed diet. EW 2: experiment 2; diet as above but weanlings initially 26–27 days old. CW 12: control weanlings for experiments 1 and 2, fed stock diet. EA: adults fed 50 per cent. *L. odoratus* seed diet. CA: control adults fed stock diet.

Fatal aortic ruptures occurred only in those animals which were initially 23–25 days old when started on the odoratus diet. The fact that aortic ruptures did not occur in the second group of weanling rats may have been due to their greater initial ages (26–27 days) at the time they commenced consuming this diet. This is in conformity with the findings of Ponseti & Shepard (1954) who showed that the incidence of aortic ruptures dropped sharply in weanling rats which started eating toxic diets when more than 28 days old. However, large and fibrosed aortic aneurysms were detected in 2 of the weanlings in the second experiment, when they were killed 216 days after starting the diets. Early changes in the spines, humeri, and especially at the knee- and wrist-joints, were striking—particularly in initially young rats (Plate, figs. A–E). Several animals were sacrificed because they developed lower limb paralyses caused by gross spinal deformities.

Microscopic findings

We consistently found both histologically and histochemically detectable changes in the aortic walls of weanling rats even within the first 14 days of the experiment. The most striking of these initial changes was the accumulation of excessive amounts of metachromatic mucopolysaccharides (MMP's) initially located primarily around the inner 2 or 3 aortic VEM's—findings confirming those of Churchill *et al*. (1955) and later of Menzies & Mills (1957). Such accumulations of MMP's were found by us to be progressive during the first 50–60 days of the experiment, during which time they became more widespread within the aortic walls and also appeared in the smaller arteries. This held only up to but not beyond the 80th day of the experiments. This reaction is consistent, being found in all weanlings, whether or not aortic ruptures supervene. In the later stages of the experiments (after 40–60 days on the diet) almost the entire thickness of the aortic wall was heavily laden with MMP's which was deposited, for the most part, immediately around but to some extent also between the collagen-like 'cores' of the VEM's previously described (Gillman, Hathorn, & Penn, 1957). Associated histochemical changes, to be described in detail elsewhere, were alterations in the amounts and distribution of resorcin-fuchsin and/or orcein-positive 'elastin', PAS-positive and ferrocyanide-positive polysaccharides, and reticulin. Elastic membranes, once ruptured, did not regenerate easily and tended to be replaced by fibrous (scar) repair of the injured areas. This observation, regarding the apparent difficulty in elastic fibre regeneration, thus seems to hold not only for arteries (Crawford, 1956; Gillman, 1957*b*; Gillman & Hathorn, 1957) but, as we have previously shown, also for the skin (Gillman & Penn, 1956).

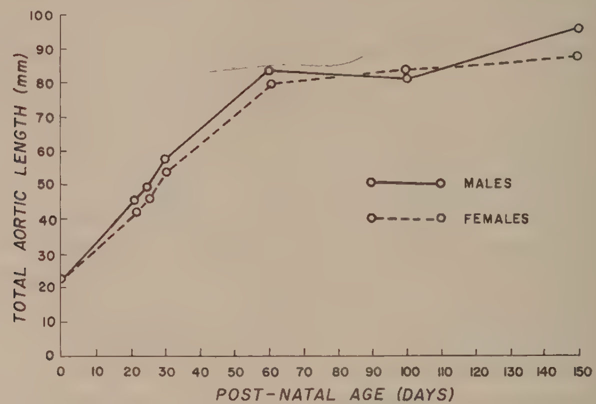
Fibroblastic proliferations, around sites of frank or incipient aortic ruptures, seemed to be characterized by initial delay in both fibrin deposition and in subsequent fibre formation. Our findings, in this regard, are in conformity with those of other workers, who, although they do not specifically remark on this absence of fibres, nevertheless all stress the 'cellularity' of the connective tissue reactions in injured areas, at least in vessels (Bachuber & Lalich, 1955) and in bones (Robinson & Bast, 1934; Ponseti & Shepard, 1954). Fibrin depositions were notably scanty at sites of frank aortic ruptures. This absence of fibrin may perhaps have been attributable to the binding of fibrinogen by circulating excess of mucopolysaccharides in a manner described by Smith & von Korff (1957). This seems all the more likely in view of the excessive amounts of histochemically demonstrable ferrocyanide-positive mucopolysaccharide seen by us in sections, lying within the lumen of blood-vessels, and particularly since we have also demonstrated a notable increase of marrow mast-cells in odoratus-fed rats (Gillman, 1957*a*). Moreover, Selye & Bois (1957) noted, in passing, that '. . . it was obvious at autopsy that clot formation was greatly delayed' (in nitrile intoxicated rats).

These disturbances in healing (viz. defects in fibrillogenesis) seem reminiscent of those found in wound healing in scorbutic guinea-pigs or in animals fed a protein-deficient diet (Udupa *et al.*, 1956) or treated with cortisone. However, the lesions in odoratism seem to differ from those found in these other experimental situations in that, in the latter, sulphation of mucopolysaccharides is apparently suppressed, whereas in odoratism it seems to be highly active. The block in fibrillogenesis, in odoratism, would thus seem to occur after that phase which is characterized by sulphation of those mucopolysaccharides which accumulate in connective tissue ground substance at sites of repair.

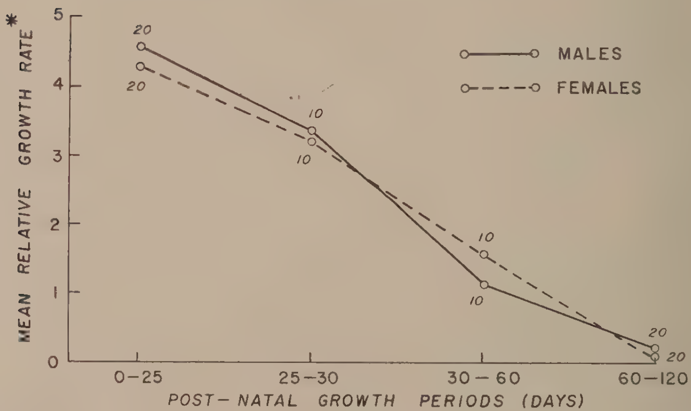
We wish to suggest a pathogenesis for the aortic ruptures different from the 'elastolytic' view maintained by others. We have accumulated evidence indicating that, during its growth the aorta normally undergoes remodelling analogous in many respects to the remodelling of long bones during their growth. The latter process is known to involve carefully integrated endosteal bone resorption occurring simultaneously with the deposition of new bone perichondrially and endochrondially at the epiphyses. Thus, although elastolysis of vascular elastic membranes does occur in weanling odoratus-intoxicated rats, this seems to be *merely part of the normal processes of aortic remodelling* accompanying the rapid growth of this vessel, especially during the first 30–40 days after weaning. The aortic ruptures, in our view, may perhaps represent the result of some disturbances of the *balance* between that lysis and regeneration of elastic membranes which probably occur simultaneously during the normal growth and remodelling of the aorta. We do not subscribe to the view outlined by Taylor (1953), and subsequently entertained by Churchill *et al.* (1955), that intramural accumulations of MMP's result from lysis or degeneration of elastic membranes. Detailed reasons for these views will be presented elsewhere.

In support of this view we found that, in normally fed rats, the aortic diameter increases by more than 40 per cent. during the first 60 days after weaning. Thereafter the aortic growth is much slower, the diameter increasing by only 25–40 per cent. (of that at weaning) between 60 and 100 days after weaning. Nor was any increase in the *total number* of aortic elastic membranes detected during growth and ageing in the rat. This perhaps implies that previously present membranes are remodelled in some way (if only at the molecular level) and replaced on a larger scale (perhaps by interstitial additions) as the aortic diameter and length increase with age and growth. The diameter of the aorta in the *experimental* weanlings increased by only 12 per cent. during the first 60 days after weaning, indicating some alterations in the normal processes of aortic growth and remodelling. Kajee (personal communication), working in our laboratories, has measured aortic growth in 70 normal male and 70 normal female rats from the day of birth to 150 days of age. Some of his findings, abstracted in Text-figs. 2 and 3, not only confirm our original observations, but have extended them in showing that the *maximal rate* of aortic growth in length in our strain of rats occurs before the 22nd post-natal day, the rate of increment

decreasing rapidly after the 30th day. These findings of Kajee may account for the great susceptibility to aortic rupture, originally described by Ponseti & Shepard and subsequently confirmed by us, of rats younger than 25 days of age



TEXT-FIG. 2. Mean total aortic length at different ages in stock male and female rats in our colony. (After Kajee, 1957.)



TEXT-FIG. 3. Mean relative growth *rates* of aortae of male and female rats in our colony over different periods. Figures within graph indicate numbers of aortae measured for each time interval. (After Kajee, 1957.)

$$* \text{ Mean relative growth rate} = \frac{\text{increment in length}}{\text{initial length} \times \text{time interval}}$$

(After Weiss, 1939.)

when first fed odoratus seeds; the rapidly progressive *decrease* in aortic susceptibility to odoratus toxin in rats of greater initial age may also perhaps be due to the smaller rate of aortic growth in slightly older animals. This observation suggests that the *rate* of aortic growth and therefore the *rate* of its associated

remodelling may in large measure determine whether or not rupture of this vessel will occur. Greater rates of growth are taken to imply speedier remodelling and therefore speedier lysis and resynthesis of structures—hence probably greater susceptibility to ruptures, should these two normally carefully integrated processes be disturbed or deranged in any way.

The above facts make understandable Ponseti & Shepard's (1954) and our own finding that the incidence of aortic ruptures decreases extremely suddenly in rats initially 28 days old as compared with rats 25 days and younger when first fed odoratus seeds. The variations in the incidence of aortic aneurysms described by other workers (Menzies & Mills, 1957) and the complete failure to produce such lesions recorded by Meyer & Vos (1956) may indeed yet be shown to be a function of the initial ages and therefore of the lesser rate of aortic growth in their experimental animals when they were first fed odoratus seeds or given the equally toxic nitriles. Thus, Menzies & Mills reported the initial weights of their rats to be 45–60 g. while Meyer & Vos stated their rats to be 33 days old (average initial weights of 70 g.). The former workers recorded only 9 instances of aortic haemorrhages, i.e. approximately 10 per cent. of rats, while the latter observers did not encounter a single instance of aortic rupture or haemorrhage in their 96 animals. All these findings are not surprising in view of Ponseti & Shepard's original report (later confirmed by us) of a profound drop in the incidence of aortic aneurysms from 75 per cent. of 50 rats which were less than 28 days old when first fed odoratus seeds, to 31 per cent. in initially 28-day-old rats. Ponseti & Shepard simply state, in a general way, that '. . . the incidence of dissecting aneurysms of the aorta was related to the age of the rat when the experimental diet was started'. Our own findings, however, lead us to suggest that the aspect of age which seems to be important in determining whether or not aortic aneurysms and other lesions will appear in odoratism (as well as their severity and speed of appearance) is the *rate of aortic growth and remodelling* during the early days or weeks of intoxication. Since this seems to be maximal in rats immediately after weaning, i.e. between 22 and 25 days old, we suggest that animals of this age should be used as standards—especially in experiments such as those which have previously been designed to determine the effects of various dietary supplements, or of hormones, on the incidence, speed of development, and course of these lesions (Strong, 1956; Selye & Bois, 1957). It is noteworthy in this regard, too, that Ponseti and Shepard reported the aortic lesions to supervene far more rapidly in younger than in older rats. In rats 22–23 days old, aortic lesions were noted as early as 12 days after the experimental diet was started; haemothorax was encountered between 34 and 56 days in 22-day-old rats as compared with 41–57 and 63–154 days in rats initially 23 and 28 days old respectively. Such observations apply primarily to the aortic lesions, but apparently not to those of the bones, since the latter seem to grow continuously in the rat.

The accumulation of MMP's in the aorta, throughout the 60-day experiments, while prominent in weanlings, is not obvious in animals which were adults when

first placed on the sweet-pea diets. Nor were excessive quantities of MMP's detectable in the aorta, even of initially *weanling* rats, after they had been on odoratus diets for 80 or more days. Such MMP's excesses may represent derangements in the regeneration of normal wear and tear injuries in aortae. The fact that this MMP's accumulation, in odoratus-fed rats, is always most marked towards the intima of the aorta, seems also to support our previous suggestion (Gillman, Hathorn, & Penn, 1957) that the rate of polysaccharide turnover in this zone of the aorta is perhaps more active than elsewhere in the vascular tree, thereby increasing the susceptibility to injuries of this part of the aorta.

Disturbances in reactivity of the connective tissues generally in intoxicated adults, or even in the older group of 'young' rats studied by us, was nevertheless evinced by the occurrence of highly cellular bone lesions, especially at sites of tendon attachments. Other evidence favouring our interpretation that the lesions in odoratism may represent the end results of derangements of fibrillogenesis (rather than fibrillo- or elasto-lysis) and of the remodelling necessitated by normal growth, are (*a*) the disturbances in fibre deposition in zones of fibroblastic proliferation associated with attempts at repair around sites of aortic injury, (*b*) the highly cellular, relatively afibrillar character of the exostoses on long bones, and (*c*) the profound disorganization of the normally integrated processes of endo- and perichondrial osteogenesis and of endosteal osteolysis both in adults and in weanlings.

Thus, our preliminary studies on the bones indicate aberrations in reticulin formation within the osseous tissue, which defect, in the organic matrix of the bones, may in part account for the ultimate deformities. This observation is in conformity with that recently reported by Menzies & Mills (1957). Profound alterations were also noted in the amount of PAS-positive material in both matrix and cartilage cells, and in the intensity and distribution of metachromasia of the matrix of epiphyseal cartilages. Also noteworthy were the striking increases in marrow mast-cells, especially in weanlings which had been on the odoratus diet for long periods (Gillman, 1957*a*).

Particularly impressive, too, especially in initially young rats fed odoratus seeds for long periods, was the gross disorganization in endosteal lysis of bone which, in healthy rats, is precisely integrated with periosteal neogenesis of bone. Thus, as shown in the Plate, figs. A–C, for the femur, two shafts were frequently noted in several long bones—the apparently older shaft *within* the bones being surrounded by new marrow, the latter in turn being enclosed within another and apparently newly deposited shaft. These changes, as well as the gross bony deformities (Plate, figs. D, E), seem to represent clear-cut proof of disorganization of remodelling processes—at least in the long bones—resulting from odoratus intoxication.

The herniae, reported by earlier workers (but not encountered in our rats) may also, perhaps, be similarly explained by derangements in fibre lysis and regeneration during remodelling of the abdominal wall.

COMMENT

Our studies of the aortic and osseous reactions in rats fed *L. odoratus* seeds seem to subtantiate our previous contention that the integrity of the collagen-like 'cores' of the vascular elastic membranes (VEM's), and possibly also of fibres in osseous matrix, is intimately dependent on their apparently spatially related and persistent reticulin and polysaccharide metabolism. The association of damage to VEM's with distinct alterations in the reticulin and mucopolysaccharide contents, around and between the aortic VEM's, confirms our previous findings in this regard (Gillman, Hathorn, & Penn, 1957). In the latter communication it was shown that *single* acute severe injuries to the media, of coronary arteries in particular, culminate, on healing, in fibrosis, tortuosity, and, with contracture of the medial scar tissue, in the ultimate stenosis of these vessels. Such repair of coronary arteries may, and we think does, predispose the now rigid, fibrosed vessels to subsequent injury of the intima and ultimately to fibrin deposits and even to thrombosis of their narrowed lumina—given certain prevailing metabolic conditions outlined elsewhere (Gillman, 1957*c*; Gillman & Naidoo, 1957, 1958; Gillman, Naidoo, & Hathorn, 1958).

It also seems possible, from our studies of odoratism, that in the aorta the most intimal of the medial elastic membranes seem to lyse first, as the aorta grows in diameter, such lysis of more intimally situated membranes being associated with the simultaneous circumferential (adventitial) formation of new membranes as the aorta grows in diameter. Such remodelling of the aorta, seen in the rat, if it occurs also in man, may account for the finding of 'abnormalities' of the intimal 'elastic' tissue, in both the coronaries and the aorta, reported even at a very early age post-natally (Schornagel, 1956; Levene, 1956 *a*, *b*). The frequently encountered 'pseudo-elastic' tissue in arteries, previously reported from this laboratory (Gillman *et al.*, 1955) and recently confirmed for human coronary arteries by Levene (1956 *a*, *b*), may also, perhaps, represent the late outcome of disturbances in the remodelling of VEM's as the delicately integrated processes, responsible for such remodelling, becomes less nicely timed with advancing age or even following metabolic disturbances.

Once the mechanisms for alterations in arterial elastic membranes, and especially those attendant on the repair of injuries, are elucidated, it may then be a relatively simple matter to account for the localization of lipid or fibrin deposits in such sites of injury on the basis of chemically and histologically definable mechanisms detailed elsewhere (Gillman & Naidoo, 1957; Gillman, 1957*c*).

Comparison of vascular and osseous reactions indicate that the *way* in which the balance between these two processes—lysis and neogenesis of structures—is disturbed can apparently vary somewhat in different loci. Thus, both in arteries and in bones, excessive tissue may be formed. This is evident from the marked thickening of the aortic wall and, in bone, by exostoses at tendon insertions as

well as by overall thickening of bone shafts. In the aorta lysis may simultaneously be highly active at one stage of post-natal development—hence the ruptures or aneurysms. In the bones, however, osteolysis may be delayed or defective at some stage and certainly the normally close integration between endosteal osteolysis and periosteal neogenesis is upset—hence the remains of old shafts within much widened marrow cavities here depicted, and associated distortions of shafts and articulating surfaces, e.g. femoral head.

In view of the apparently widespread connective tissue derangements here disclosed, the rate of growth and remodelling of arteries and bones and their apparent dependence on connective tissue carbohydrate metabolism, it is anticipated that the application of toxic nitriles, together with the use of radioisotypes, will be invaluable in testing our interpretations. Such studies may also prove most useful in unravelling the mechanisms whereby amino acids are incorporated into the protein chains of fibres as well as the relation between polysaccharide metabolism and fibre synthesis, especially in connective tissue throughout the organism at different stages of growth and maturation.

SUMMARY

1. Evidence is presented in support of the view that lesions in vessels, bones, and perhaps in connective tissues generally, induced by feeding *L. odoratus* seeds to rats, may be attributable primarily to some disturbance in the neogenesis of fibres during the growth, regeneration, and/or repair of connective tissues. This failure seems to be at a stage after sulphation of those mucopolysaccharides which accumulate in connective tissue ground substance during fibre formation. It is suggested that the aortic ruptures may be the result of some *derangement in the balance* between lysis and regeneration of elastic membranes, which probably occur simultaneously during the normal growth and remodelling of the aorta. This interpretation differs from that provided by other workers who attribute aortic ruptures solely to elastolysis. Evidence from the study of lesions in bones and alterations in repair processes around the aorta in *L. odoratus*-fed adult and weanling rats supports the interpretations here presented. The frequency of 'double-shafts' as well as other deformities of long bones are taken as clear indications of disorganized remodelling of bones during growth.

2. These studies of the aortic reactions in rats fed *L. odoratus* seeds also substantiate our previous contention that the integrity of the collagen-like 'cores' of the VEM's is probably intimately dependent upon their 'sleeves' of reticulin and polysaccharides. The association of damage to the elastic membranes with distinct alterations in the reticulin and mucopolysaccharide contents immediately around and also between aortic VEM's confirm our previous findings in this regard.

3. There are indications, too, from histochemically detectable increases in circulating mucopolysaccharides, from the marked increases in osseous

mast-cells and from reported delay in blood-clotting, that widespread disturbances in polysaccharide metabolism prevail in odoratus-intoxicated rats.

4. Some possible implications of these findings for understanding the genesis of human arterial lesions are briefly discussed with special reference to a developmental approach to the aetiology and pathogenesis of these vascular reactions in terms of general features of connective tissue regeneration and repair.

ACKNOWLEDGEMENTS

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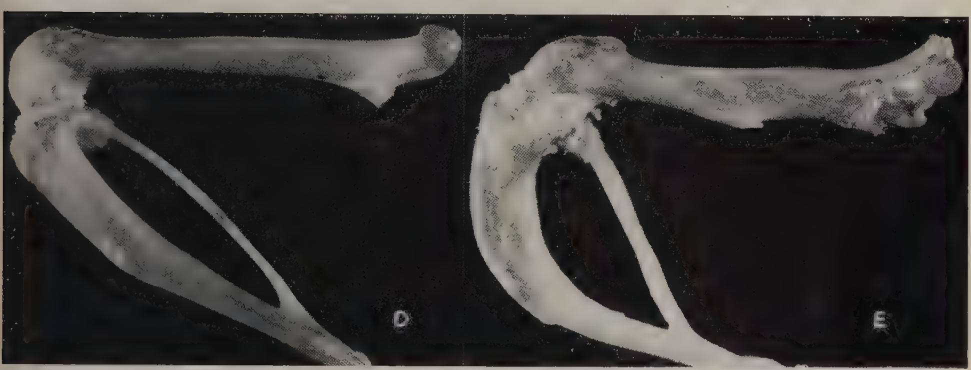
EXPLANATION OF PLATE

FIGS. A, B, C. Longitudinal sections of upper ends of femora. A from control rat to compare with B and C which are serial sections of the same femur of an initially weanling rat after consuming *L. odoratus* seeds for 103 days. Note in B and C the distortions of the head and neck, the difference in endosteal trabeculae in neck and great trochanter, the delayed resorption of old neck bone within marrow (in B), the dual shaft and marrow cavity (in C), and the extremely thin periosteal bone in B and C as compared with A; all these being indicative of disturbed osteal modelling and growth. ×3



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FIGS. D, E. Macroscopic appearances of several lower limb bones of two, initially weanling, rats when 198 days old. D: control; E: fed *L. odoratus* seeds for 174 days. Shows in intoxicated rat gross distortions of femoral, tibial, and fibular shafts and especially their great widths (compare also with figs. B and C), exostoses at muscle insertions, and disturbed proportions, e.g. of femoral head, neck, trochanters, and condyles. ×2.

(Manuscript received 19 : viii : 57)

Teratogenic Effects of Trypan Blue on Hamster Embryos[1]

by VERGIL H. FERM[2]

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WITH ONE PLATE

LITTLE information is available in the literature on the susceptibility of the hamster embryo to teratogens. Desoxycorticosterone acetate apparently has some teratogenic activity (Tedford & Risely, 1950), but the degree and type of abnormality produced by this agent in the hamster have not been recorded. Orsini (1952) has described naturally-occurring urogenital anomalies which appear in females of a piebald strain of the golden hamster.

The short gestation period, the accuracy of timed matings (Ward, 1946), and the large litter size make this animal an excellent one for embryological research on teratogens. The purpose of the present study was to determine the susceptibility of the hamster to the teratogenic effects of trypan blue and to learn what effects this dye had on other aspects of pregnancy and gestation. Neither the site nor the mode of action of this compound has been established. Failure to detect the dye in the embryonic tissues proper has led to hypotheses suggesting that some derangement of maternal physiology (Gillman *et al.*, 1948) or alterations in the permeability of the yolk-sac placenta, because of its peculiar affinity for the dye (Hamburgh, 1954), was responsible for the teratogenic activity. Observations on the placental transfer of trypan blue into the fluid of the young rabbit blastocyst (Ferm, 1956) introduce the possibility that the dye may have a direct teratogenic effect on the developing rabbit embryo.

MATERIALS AND METHODS

The variables of pregnancy measured in this experiment included: gross foetal weights, maternal weight gain, placental weights, placental–foetal weight ratios, total litter size, percentage of litter resorption, and the survival sex ratio.

Adult female golden hamsters (*Cricetus auratus*), from a colony maintained in this laboratory by Dr. Margaret Orsini, were used in this experiment. At least two estrous cycles were followed in all the animals. Sixty hours prior to the

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estimated time of the next succeeding ovulation, the animals received subcutaneously one-half of their total dose of trypan blue (obtained from Matheson Coleman & Bell, Co., Norwood, Ohio) as a 1 per cent. solution in saline. This preovulation injection was given to observe the effect of the dye on ovulation. Forty-eight hours after injection the females were put with healthy, rested males from 4 p.m. to 8 a.m. The vaginal discharge was examined the morning after mating for the typical post-estrous appearance (Deanesly, 1938). On the fifth day of gestation (about 130 hours after the estimated time of ovulation) the females received the remaining half of the dose in an identical manner. One-half of the experimental group received a total of 10 mgm. of the dye (Series X), the other half received 15 mgm. (Series XV). Control animals received $\frac{1}{2}$ c.c. of normal saline at corresponding times before and during pregnancy. On the fifteenth day of gestation (developmental age of 14 days, 8–9 hours), the females were sacrificed and the foetuses and their placentae removed, examined, and weighed on a Roller-Smith torsion balance. Resorption sites were carefully sought and included in the record of total litter size. Maternal weights were recorded at the time of the first injection and again at the time of sacrifice. All animals were individually caged from the time of the first injection and were handled minimally. The diet consisted of Purina laboratory chow supplemented twice weekly with fresh greens.

RESULTS AND DISCUSSION

The results of this experiment are summarized in Table 1 and the evidence of anomalies may be found in figs. 1–4 of the Plate.

Under the conditions of this experiment, trypan blue is a teratogenic agent in

TABLE 1

Effect of trypan blue on hamster embryos

Series	*No. of animals*	*Maternal weight gain (gm.)*	*No. of living foetuses*	*Per cent. resorption*	*Mean foetal weight (mgm.)*	*Mean placental weight (mgm.)*	*Placental-foetal weight ratio*	*Sex ratio*	
								Female	*Male*
Controls	14	28·6	132	9·4	1,425 ±22·5*	446 ±13·9*	0·312	66	48
Series X	12	29·5	58	58·0	1,248 ±13·7*	442 ±12·1*	0·354	29	20
Series XV	12	25·2	39	73·7	1,202 ±15·4*	390 ±11·5*	0·324	18	15

* Standard error.

hamsters as evidenced by the finding of anomalies in 13 foetuses from 8 litters of the total of 24 treated litters of the experimental groups. Only one of the 14 control litters contained anomalies. The teratogenic effects were apparently limited to the cephalic axial region, though careful internal dissections or serial microscopic sections were not done. Anomalies noted were hydrocephalus,

encephalocoeles, and exencephaly. There was one litter of nine hydrocephalic foetuses in the control series. No reference to this type of anomaly in hamsters could be found in the literature, and it has not been noted in the numerous stock animals used in other experimental work in this laboratory.

There appears to be no effect of the dye on the number of ova ovulated or fertilized, or on the number of blastocysts that begin implantation, since the mean litter size, including both the living foetuses and the observable resorption sites, is nearly equal in the control (11·3) and experimental (12·0) groups. This, however, does not preclude a possible sublethal toxic effect of the dye directly on the ovum in the follicle or in the early cleavage stages. There was a marked effect on the intrauterine resorption of implanted blastocysts. This probably occurred well after implantation began since resorption was recognized by macroscopic means. Well-developed placental tissue (chorio-allantoic) was found in most of these resorption sites and, in one case, a well-preserved limb-bud embryo was found at the time of sacrifice, which was one day before term. The placenta of this animal was as large as placentae of the control series of the same developmental age.

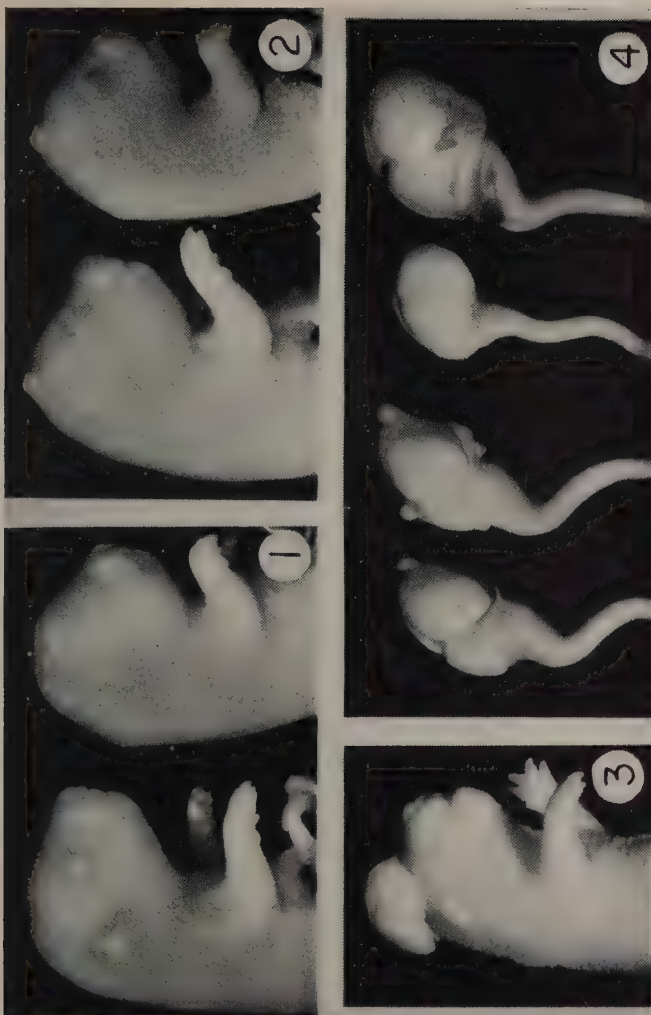
There is a decided effect of trypan blue on foetal weight. The foetuses of the experimental series weighed less than did the controls, and this decrease in weight appears to be in proportion to the amount of dye given the mother. The effect of the dye treatment on placental weights and on the placental-foetal weight ratios was less striking. The observed sex ratios of the living foetuses showed that there was no significant sex susceptibility to the dye.

The vitelline membrane of all the experimental foetuses was stained a deep blue colour and those from animals receiving larger doses of the dye had a more intense coloration. By visual examination, all placentae of a single litter appeared to stain with the same degree of intensity. The finding of litters in which only one or two of the foetuses were malformed and in which all of the vitelline membranes appeared to be stained with the same intensity, tends to diminish the possibility that the teratogenic action of the dye occurred through an alteration in the permeability of the yolk-sac placenta. The failure of the dye to affect maternal weight gain during pregnancy reduces the possibility that a maternal metabolic disturbance is responsible for the teratogenicity of trypan blue. The remaining possibility is a direct toxic action on the developing embryo.

SUMMARY

1. In a series of 24 treated litters of hamsters, trypan blue was a teratogenic agent, causing malformations mainly in the cephalic axial region. The dye had no effect on the number of ova ovulated, the number of blastocysts that began implantation, or the maternal weight gain during pregnancy.

2. Embryos of the experimental series weighed less than those of the control series injected with saline only, but trypan blue had little effect on placental weights or placental–foetal weight ratios.



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3. A naturally occurring hydrocephalic anomaly was found in the control stock.

4. It is suggested from these data that trypan blue has a direct teratogenic effect upon the embryo.

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EXPLANATION OF PLATE

FIG. 1. Foetus at left is a normal one from a control litter. Hydrocephalic foetus at right is from a mother of Series XV. Hydrocephalic head is enlarged and rounded. $\times 3$. See fig. 4.

FIG. 2. Hamster litter-mates from a Series X mother. Both show a small dorsal midline evagination which arose from the mid-brain region. $\times 3$. See fig. 4.

FIG. 3. Foetus from a mother of Series XV, showing a well-developed encephalocoele. This foetus was living at the time of sacrifice and the amniotic fluid was grossly bloody. $\times 2$. See fig. 4.

FIG. 4. Brains and upper spinal cords of selected typical anomalies. From left to right: (1) normal control animal, (2) small dorsal encephalocoele, (3) brain removed from an exencephalic foetus, (4) brain removed from a typical hydrocephalic foetus showing marked dilatation of the cerebrum with thinning of its walls. $\times 3$.

(Manuscript received 13 : ix : 57)

Epithelial Regeneration and Wound Healing in the Oesophagus of the Cat

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From the Department of Anatomy, University of Sheffield

WITH THREE PLATES

INTRODUCTION

SAINT in 1929 documented the experimental studies that had been carried out up to that date on the healing of oesophageal wounds. The reported observations dealt with the relatively long-term results, and little attention was paid to the early changes that occurred during the period immediately following the infliction of the wound. Recently, Malm (1951) made observations following surgical experiments on the oesophagus in dogs, but again no reference was made to the early changes in epithelium or connective tissue. The most recent experimental work on oesophageal healing appears to be that of Picard, Henry, Cotte, & Inglesakis (1956), but their interest lay in the repair of muscular tissue.

The present investigation was designed to study the behaviour of epithelium and connective tissue in the cat's oesophagus, following the removal of small areas of mucous membrane. Routine histological methods were supplemented by a series of histochemical studies on both the normal and regenerating tissues.

MATERIALS AND METHODS

All operations were carried out on healthy adult cats, a total of 31 animals being used.

Operative procedure

Under anaesthesia with intraperitoneal nembutal, the lower end of the oesophagus (i.e. the abdominal part) was approached through an upper abdominal midline incision. The oesophagus was opened by a longitudinal incision in its ventral wall and a piece of mucous membrane about 0·5 cm.^{2} in size was removed. The oesophageal wound was closed by a single continuous catgut suture which did not incorporate the mucosa, and the abdomen closed in layers. Following operation the animals were allowed to survive for periods ranging from 6 hours to 1 year. During the immediate post-operative period, the animals

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were fed with water alone on the first day and milk alone on the second and third days; normal feeding was then resumed.

Histological and histochemical techniques

After death by coal-gas poisoning, the oesophagus was opened and the site of the lesion was excised, pinned out on cork, and fixed either in ice-cold 80 per cent. alcohol or in ice-cold Carnoy's fluid. The tissue was embedded in paraffin wax and serial sections were cut at a thickness of 8 μ; every 20th was mounted and stained with haematoxylin and eosin. These sections were then examined and from regions of particular interest serial sections were mounted for histochemical studies. For the demonstration of alkaline phosphatase the Gomori cobalt sulphide technique was employed, the substrate being sodium-β-glycerophosphate, and the incubation periods ranging between 15 minutes and 24 hours. The findings with this technique were confirmed with the azo dye method. The periodic acid-Schiff (PAS) technique was used for the demonstration of polysaccharides, glycogen being identified by comparison of sections treated or untreated with saliva. The presence of ribonucleic acid (RNA) was demonstrated by the methyl green pyronin technique (Kurnick, 1955), and metachromasia by the use of toluidine blue (0·5 per cent. for 30 minutes) or of azure A (0·1 per cent. for 10 minutes).

RESULTS

The normal oesophageal mucosa

The epithelium in the lower end of the cat's oesophagus is of the stratified squamous variety in which there is no keratinization of the surface cells. Sections stained with haematoxylin and eosin (Plate 1, fig. A) indicate that this epithelium consists of three zones of cells, viz. basal, intermediate, and superfical. The basal zone consists of 2 to 3 rows of small polygonal cells whose nuclei stain densely. The intermediate zone varies considerably in thickness, consisting of many layers of cells where the epithelium protrudes into the lamina propria, and is formed by large polyhedral cells with faintly staining cytoplasm and nuclei. The superficial zone comprises several layers of flattened cells whose nuclei appear pyknotic. This differentiation of the epithelium into three zones can be further demonstrated following histochemical procedures. The reaction for alkaline phosphatase, using the Gomori technique with short incubation periods of 15 to 30 minutes, was confined to the superficial and basal zones, the intermediate zone being invariably negative (Plate 1, fig. B). This appearance was similar with the azo dye method (Plate 1, fig. C), from which the impression was formed that the reaction was confined to the cell-membrane, or intercellular substance, particularly in the basal zone. Staining with toluidine blue again emphasized the division into the three zones by the fact that they stained with different intensities (Plate 1, fig. D). In addition to demonstrating the zoning of the epithelium, toluidine blue (and azure A) revealed the presence of a large number of mast

cells. These were found not only in the lamina propria, where they were usually associated with blood-vessels, but also in the basal zone of the epithelium (Plate 2, fig. G). In no specimen were any mast cells seen in the intermediate or superficial zones. They varied considerably in size and in the extent to which their cytoplasm was filled with metachromatic granules. From the study of the present series it appeared that the number and position of the mast cells in the normal epithelium of the lower part of the oesophagus varied little from one animal to another.

Sections treated by the PAS technique demonstrated that the normal epithelium did not contain any histochemically detectable glycogen. PAS-positive material other than glycogen was concentrated in the superficial zone, and appeared to be intercellular (Plate 1, fig. E).

When stained by the methyl green pyronin technique for RNA, the normal epithelium gave a strongly positive reaction in the intermediate zone, a faintly positive reaction in the superficial zone, and no reaction in the basal zone (Plate 1, fig. F).

The healing wound

Six hours after operation the site of the lesion (Plate 2, fig. I) could be recognized macroscopically by the absence of mucosa and the presence of blood and fibrin clot. During subsequent days reddish granulation tissue was seen, and by the end of 2 to 3 weeks a white, slightly puckered area offered naked-eye evidence of the region of operation. The sites of lesions after 3 and 6 months and 1 year could only be recognized microscopically after sectioning what was considered to be the area concerned.

Epithelial repair

Within 24 hours epithelial cells had begun to spread or migrate from the wound margin over the floor of the lesion. The migrating cells were several layers thick and no longer displayed the normal zoning. By the end of 2 days this migration had increased and there was considerable mitotic activity in the surrounding epithelium. Any high-power field of epithelium adjacent to the ulcer margin showed several nuclei in different stages of mitosis (Plate 2, fig. H), a state in striking contrast to the normal epithelium where very few mitotic figures were seen.

The cells of the spreading epithelium and those at the margin of the wound were considerably larger than normal, a feature particularly marked when normal basal cells were compared with those that now formed the basal layer of the spreading epithelium. Mast cells were never found among the migrating cells.

From the third to the tenth day the process of epithelial migration accompanied by mitotic activity continued. During this period the epithelium became infiltrated by polymorphonuclear leucocytes and lymphocytes. These cells were

concentrated chiefly in the upper layers and many were found in spaces which made their appearance during this time in these regions (Plate 2, fig. J). The spaces in which white cells were seen varied greatly in sizes and gave a degenerative appearance to the epithelium. They were never seen in the basal layers.

By the tenth day the ulcer floor had become completely epithelialized (Plate 2, fig. K) and the increased mitotic activity had now declined. At this stage the cells were still considerably larger than normal, and in the more superficial regions some inflammatory cells and spaces were still found (Plate 2, fig. L).

Subsequent stages in the epithelial repair consisted of a thinning of epithelium due to a decrease in the size of the cells, and a return to the zoning seen in the normal epithelium, with an absence of infiltration. The normal pattern had been reestablished within 1 month (Plate 2, fig. N), and by this time mast cells were again noted in the basal layer of the epithelium now overlying the original wound area.

The normal distribution of alkaline phosphatase was not seen in regenerating epithelium; the enzyme was completely absent from the migrating cells, even up to the time of complete epithelialization (Plate 3, fig. P). However, when the stratified pattern of the epithelium was becoming restored (during the second week), a reaction for the enzyme occurred in the superficial zone (Plate 3, fig. R). From the third week onwards alkaline phosphatase could be demonstrated in the basal region also; thus the reaction pattern seen in undisturbed epithelium had now returned (Plate 3, fig. T).

Alterations in the distribution of PAS-positive material were also noted in the regenerating epithelium. The hypertrophic cells at the wound margins, and those that were migrating, contained abundant quantities of glycogen, with the notable exception of cells that formed the most basal layer, which never contained this substance (Plate 3, figs. O and Q). The PAS-positive material other than glycogen, which was a prominent feature of the superficial zone of undisturbed epithelium, was no longer present in cells at the margin, nor was it found in those that were spreading over the floor of the wound (Plate 3, fig. S). Following complete epithelialization and the reappearance of stratification, glycogen could no longer be detected histochemically, but non-specific mucopolysaccharide could be demonstrated again in the superficial zone (Plate 3, fig. U).

With the methyl green pyronin stain there did not appear to be any significant increase in pyroninophilia in the regenerating cells.

Connective tissue reactions

The changes that occurred in the floor of the lesion were essentially those of the accumulation and maturation of granulation tissue, together with a considerable degree of infiltration with inflammatory cells. Mitotic activity in connective tissue cells was first noted on the second post-operative day, and was most marked by the fourth or fifth day after which it gradually declined. Metachromasia of the ground substance in the wound area became prominent by the

end of the first week, was still present at the time of complete epithelialization, and thereafter disappeared. Mast cells were absent from the wound site until about the end of 1 month, when a few could be detected in the vicinity of blood-vessels.

At all stages of the healing process there was a complete absence of alkaline phosphatase in the newly forming connective tissue (Plate 3, fig. R). This was so even after prolonged incubation periods of up to 24 hours, when all the tissue elements, including dividing fibroblasts, young blood-vessels, and fibrous extra-cellular material, still gave a negative reaction.

After a number of months some wound areas remained relatively flat (Plate 2, fig. M) while others developed a convoluted pattern more closely resembling the architecture of normal mucosa and submucosa. In all wounds the muscularis mucosae showed no evidence of regeneration and the cut edges remained to give some indication of the margin of the original lesion (Plate 2, fig. M).

DISCUSSION

The results show that, following repair, the site of a small oesophageal wound eventually bears a close resemblance to the normal mucosa, the muscularis mucosae being the only tissue that shows no evidence of restoration. The absence of regeneration in this component of the alimentary wall has been repeatedly observed both in animals and in man (Ivy, Grossman, & Bachrach, 1952).

The repair process in oesophageal epithelium resembles that in epidermis, the tissue upon which the majority of studies on wound healing have been carried out. In both instances there is a migration of cells from the wound margin, with hypertrophy of the cells concerned, followed by increased mitotic activity in the surrounding epithelium. No convincing evidence of the presence of mitosis in migrating epithelial cells was found in the present work. This contrasts with comparable studies made by the authors on regenerating urinary bladder and gall-bladder epithelia (McMinn & Johnson, 1955, 1957), where cells in division could be found without difficulty among those that were migrating. The infiltration of new oesophageal epithelium by inflammatory cells and the presence of spaces which lend to it a degenerative appearance are phenomena not shared with epidermis, but they may be due to the relatively septic environment of the upper part of the alimentary tract compared with the skin. The fact that the inflammatory invasion was less pronounced in the basal layers of cells may indicate that the nutrition and metabolism of these basal cells is less disturbed than that of more superficial cells which are farther removed from the sub-epithelial tissue fluid, their probable source of nutriment.

The presence and distribution of glycogen in the regenerating epithelium is also comparable with the findings in hypertrophic epidermis (Bradfield, 1951), including the absence of glycogen from the basal layers of both types of epithelia. It appears that in the oesophagus there is a reciprocal relationship between the

presence of glycogen and of other polysaccharides. When cells are migrating the PAS-positive material normally present in the superficial zone of the epithelium disappears, and glycogen is then found in most of the enlarged cells; when the glycogen disappears, the PAS-positive material returns to the upper layers of cells. Wislocki, Fawcett, & Dempsey (1951) consider that such PAS-positive material in squamous epithelia is concentrated in the intercellular spaces and on cell surfaces, and that its presence varies inversely with the amount of keratin in any given epithelium. Since the epithelium of the cat's oesophagus is non-keratinizing, the present finding of PAS-positive material in the superficial zone is in accord with the observations of these workers. Scothorne & Scothorne (1953) have reviewed the possible reasons for glycogen accumulation in epidermal cells and concluded that none of the theories so far advanced is entirely satisfactory.

The finding of alkaline phosphatase in the deep and superficial layers of the normal epithelium is unexpected. In epithelia, e.g. of the intestine and renal tubules, its presence has been correlated with absorptive processes, but the oesophageal lining is not regarded as a tissue through which the passage of solute normally occurs. No reason can be given for the absence of the enzyme among the migrating cells; a number of as yet unknown physico-chemical changes may be occurring in the cell and in the intercellular substance in order to allow cells the freedom to move, but it is not possible to say whether the absence of phosphatase is correlated with such changes.

The regularity with which large numbers of mast cells occur in the basal layer of normal oesophageal epithelium in the cat has already been reported (Johnson & McMinn, 1957) but Mota, Ferri, & Yoneda (1956), who investigated the distribution of mast cells in the digestive tract of laboratory animals (including the cat), do not mention them as being a feature of the epithelium.

The reactions in the connective tissue of the floor of the lesion are in general typical of those of any wound that is healing by granulation. Although mast cells have been considered to play some part in the formation of new connective tissue, particularly the metachromatic ground substance (Asboe-Hansen, 1954), their absence from the wound area does not support this hypothesis. In this respect the present work is in accord with the results reported by Taylor & Saunders (1957) who studied the fibrogenesis occurring around implants of gelatine sponge in rats; mast cells were again notably absent from the developing and maturing granulation tissue.

The absence of alkaline phosphatase in the newly forming connective tissue elements is very striking and contrasts with the results of work carried out by Fell & Danielli (1943) and others, who noted in the healing skin wounds of rodents a considerable increase in phosphatase activity. However, it is of further interest that in the study of healing gall-bladder wounds in the cat, McMinn & Johnson (1957) were again unable to demonstrate the presence of phosphatase. The fact that a species difference may account for the conflicting results found

in cats and rodents has led to further work on this subject which will be presented elsewhere (Johnson & McMinn, 1958).

SUMMARY

1. Histological and histochemical techniques have demonstrated that the epithelium in the lower end of the oesophagus of the cat consists of three well-defined zones, namely superficial, intermediate, and basal.

2. Wound healing in the lower end of the oesophagus has been studied following the removal of small areas of mucosa.

3. Epithelial repair occurred by migration of cells, accompanied by increased mitotic activity in the surrounding undisturbed epithelium.

4. The alkaline phosphatase and PAS-positive material (other than glycogen) that were present in normal epithelium were not seen in migrating cells, which contained considerable quantities of glycogen.

5. The newly forming connective tissue elements in the floor of the wound did not contain alkaline phosphatase at any stage of the healing process.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Normal oesophageal epithelium. Note the small basal cells, the larger cells of the ntermediate zone, and the layers of flattened cells that form the superficial zone. There is no eratinization. Haematoxylin and eosin. ×170.

FIG. B. Normal epithelium, showing the presence of alkaline phosphatase in the basal zone, nd to a lesser extent in the superficial layers. Gomori technique, incubation time 30 minutes. ×170.

FIG. C. Normal epithelium, showing a similar distribution of alkaline phosphatase to that in ig. B. Azo dye method. ×170.

FIG. D. Normal epithelium, showing the three zones of cells. Toluidine blue. ×170.

FIG. E. Normal epithelium, showing PAS-positive material in the superficial zone. PAS after aliva digestion. ×170.

FIG. F. Normal epithelium. Note that pyroninophilia is most marked in the intermediate zone nd absent from the basal layer. Methyl green pyronin. ×500.

PLATE 2

FIG. G. Normal epithelium, showing mast cells lying in the basal layer. The granules are trongly metachromatic. Azure A. ×540.

FIG. H. Normal epithelium near the wound margin after 2 days, showing a number of mitotic igures. Haematoxylin and eosin. ×500.

FIG. I. Section through the centre of a wound 6 hours after operation. Note that the whole hickness of the mucosa including the muscularis mucosae has been removed. Haematoxylin and osin. ×25.

FIG. J. Epithelial cells migrating towards the right over the floor of the wound, after 3 days. Note the size of the basal cells compared with fig. A, the cellular infiltration, and the spaces in the ipper layers. Haematoxylin and eosin. ×170.

FIG. K. Completely epithelialized wound site after 10 days, showing the thickened epithelium overlying maturing granulation tissue. Haematoxylin and eosin. ×30.

FIG. L. Hypertrophic epithelium overlying a wound site after 10 days at the same magnification is figs. A and J. Note the absence of zoning. Haematoxylin and eosin. ×170.

FIG. M. Section through a wound site 6 months after operation. Note the cut edges of the nuscularis mucosae and its absence from the flattened wound area. Haematoxylin and eosin. ×25.

FIG. N. Epithelium from a wound area 1 month after operation, showing the return of the iormal zoning. Compare with figs. A and L at the same magnification. Haematoxylin and eosin. ×170.

PLATE 3

FIG. O. Epithelium from a wound margin after 3 days, showing abundant quantities of glycogen, but note its absence from the basal layer. PAS. ×750.

FIG. P. Epithelium from an epithelialized wound after 10 days. Compare with fig. B and note the absence of alkaline phosphatase from the epithelium. Gomori technique, incubation time 30 minutes, lightly counterstained with haematoxylin. ×120.

FIG. Q. An adjacent section to that illustrated in fig. O, showing that the material identified as glycogen in fig. O has now been removed. PAS after saliva digestion. ×750.

FIG. R. Part of an epithelialized wound area after 15 days. Compare with figs. B and P and note that the superficial zone of epithelium gives a reaction for alkaline phosphatase, and that the subepithelial granulation tissue gives a completely negative reaction. Gomori technique, incubation time 30 minutes, lightly counterstained with haematoxylin. ×120.

FIG. S. Section of a wound margin after 10 days. Normal epithelium, above, gives a PAS-positive reaction in the superficial zone. The large, migrating epithelial cells, below, contain glycogen. PAS. ×120.

FIG. T. Epithelium from a healed site after 6 months, showing a normal distribution of alkaline phosphatase. Gomori technique, incubation time 30 minutes. ×170.

FIG. U. Epithelium from a healed site after 6 months, showing a normal distribution of PAS-positive material other than glycogen. Compare with fig. E. PAS after saliva digestion. ×170.

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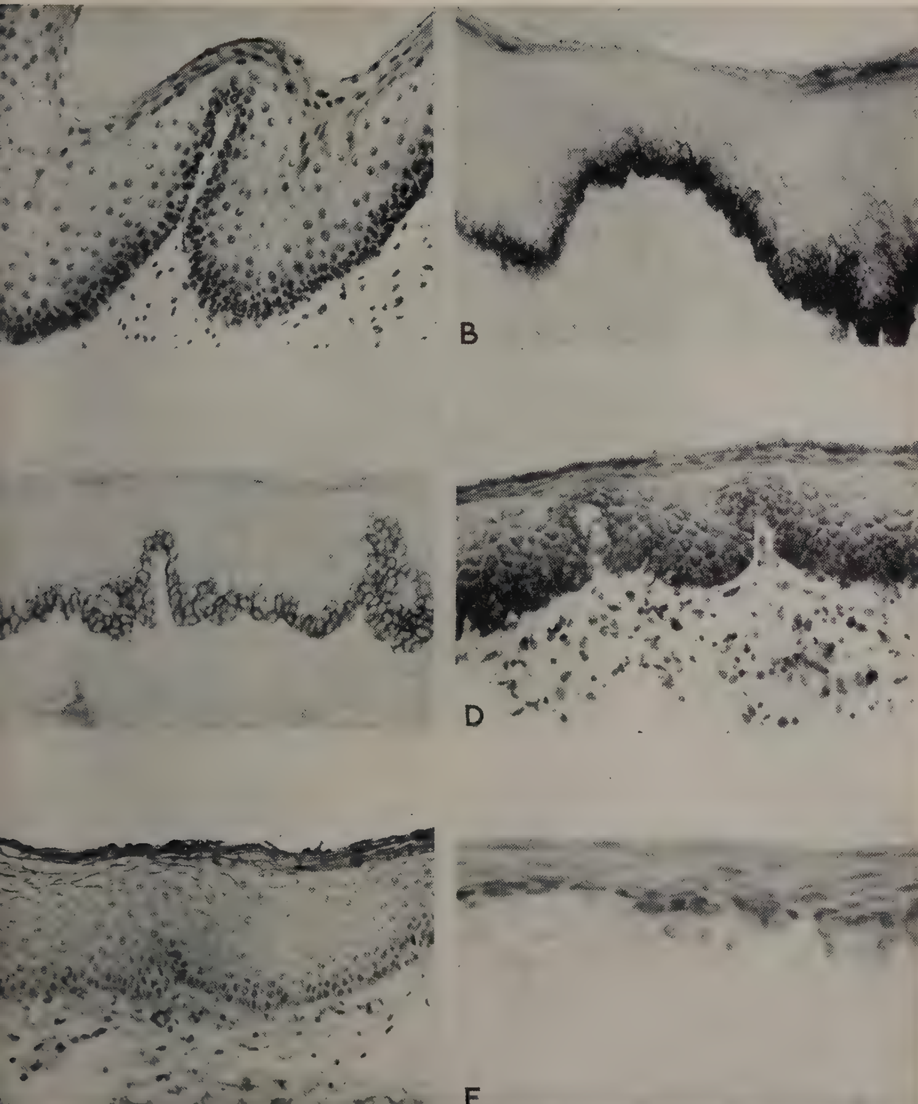

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Plate 1

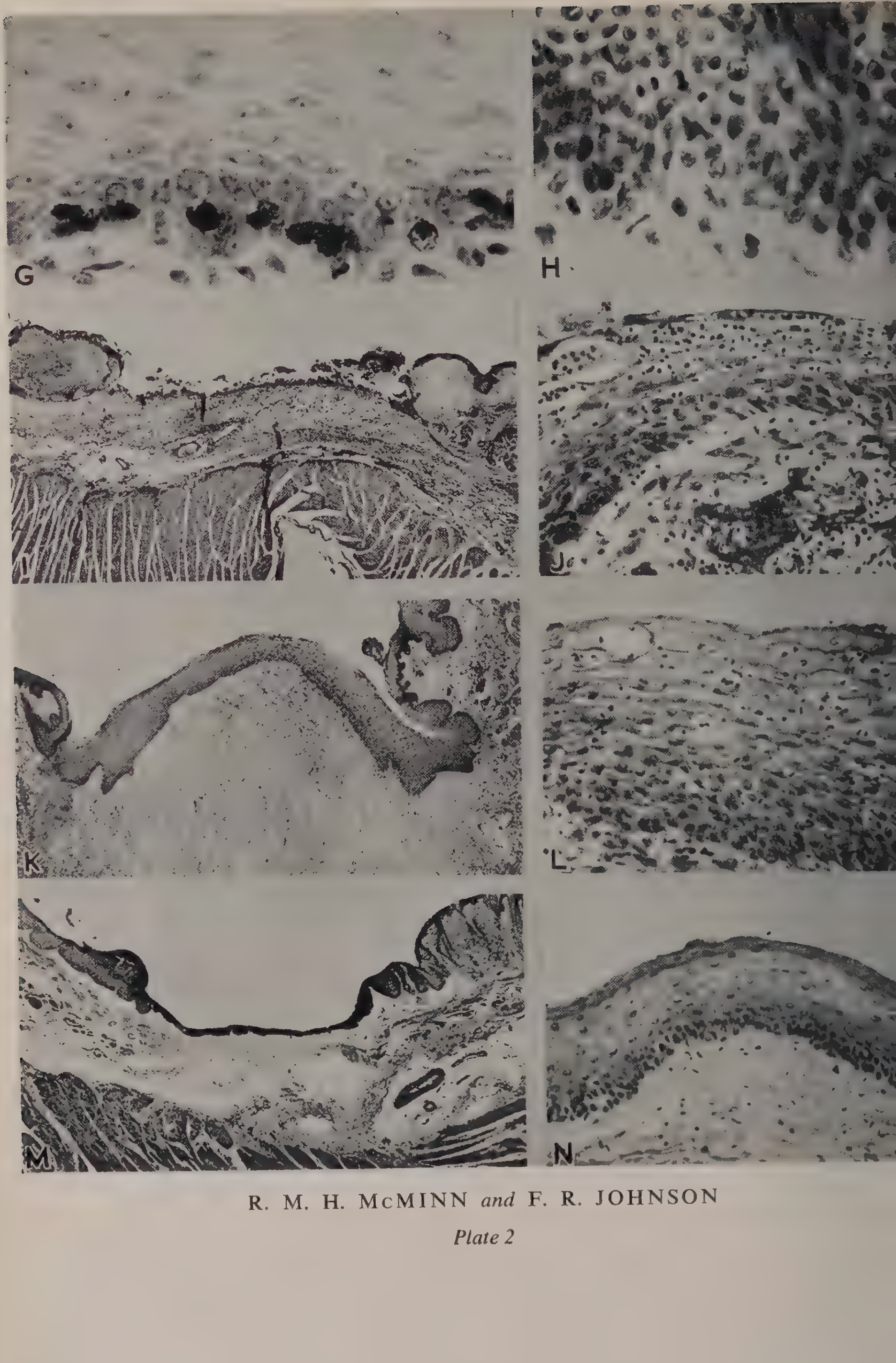

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Plate 2

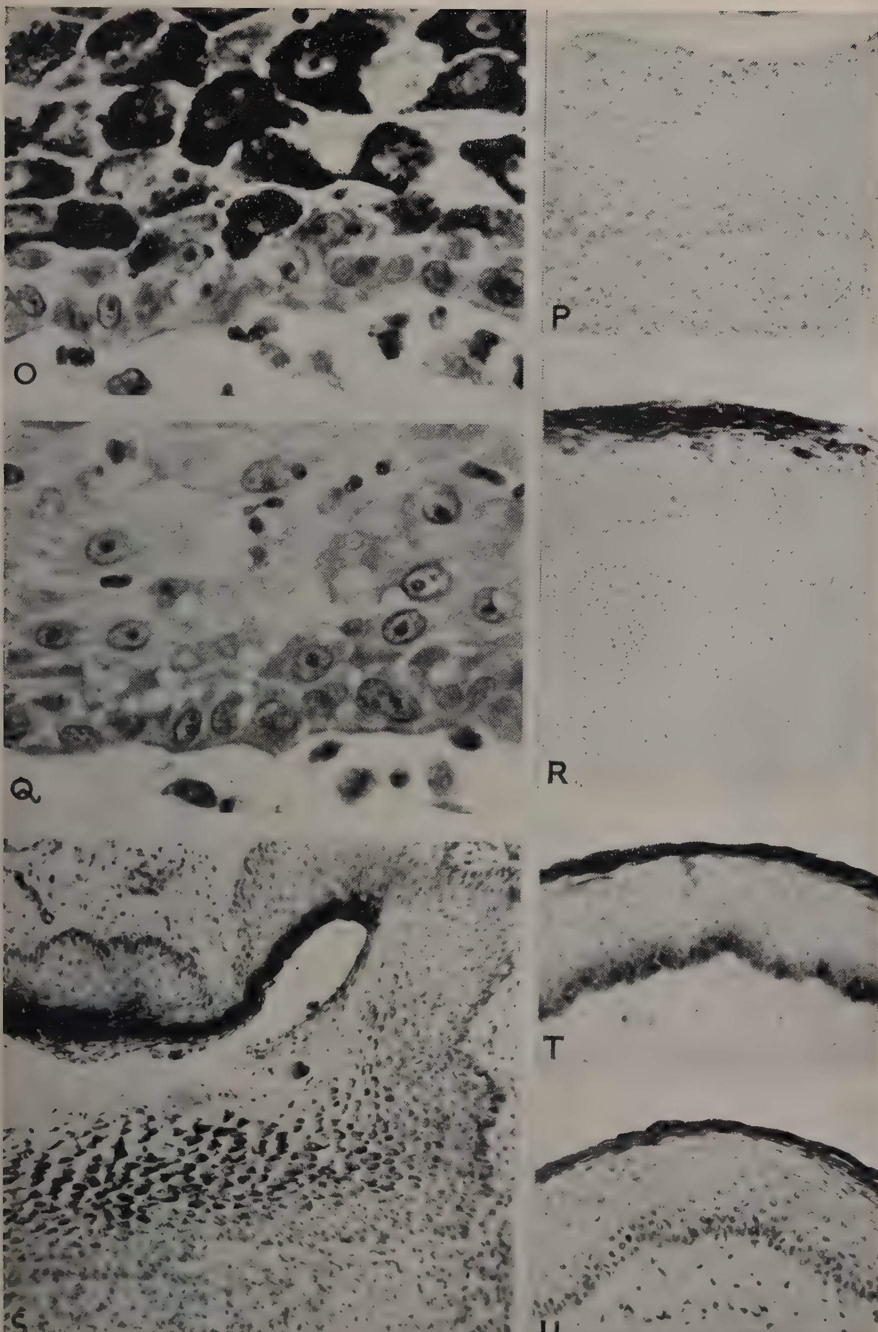

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Plate 3

On Regeneration after the Amputation of Abnormal Structures

I. Defective Amphibian Tails

by D. R. NEWTH[1]

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INTRODUCTION

WHEN the loss of an animal member is followed by its regeneration the new structure is not necessarily a replica of the old. It may, for example, be an anatomically inadequate substitute, or hypomorph. In certain cases it is recognizably the equivalent of some other member characteristic of the species in question—a heteromorph. Systemic and environmental influences operating during the process of regeneration sometimes play an important part in deciding what is to be formed, but so, too, of course, do the properties of the cells of the blastema and stump (for review see A. E. Needham, 1952). These cells, and their forebears, have all had the experience of being part of an organism undergoing primary embryogenesis, but it is far from clear to what extent this experience has relevance for the successful completion of regeneration.

It is true that the very capacity to regenerate at all is a product of normal development and usually arises only after embryogenesis is accomplished, but this is another matter. Once given the ability to regenerate, is the information contained within the cells of the blastema that guides their morphogenesis inherited without change through the cell lineage which connects them to the fertilized egg, or is part of it acquired during embryogenesis?

So long as regeneration is studied only where it follows the removal of normal structures this question must remain unanswered. But structures whose primary development has been abnormal offer us the possibility of finding out, to put it in an extreme and simple form, how far regeneration is a process tending to the reproduction of what *was* there and how far to the production of what *should* have been there. It is unlikely that the answer will be the same in every case. Indeed, we know that it will not be, for a white axolotl (i.e. one homozygous for the gene *d*) is, in one sense, abnormal and will replace a lost hand by another white one, whereas transplanted limb disks may give rise to malformed limbs

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which, on amputation, can be replaced by normally formed ones (Harrison, 1918; Swett, 1924).

It is clear that abnormalities of different kinds will have different meanings for this problem. Unfortunately no classification of them can be wholly satisfactory. Thus the following provisional scheme cannot claim to be based upon mutually exclusive categories with a fundamental status in teratology, since only in extreme cases will an abnormality lie almost entirely within one category alone. At best it can help to make possible an orderly approach to the relationship of regeneration to abnormal development.

A. *Inherited abnormality*

A.1. *Chromosomal defects* whose origin may lie a few or many generations before the fertilization of the zygote in which they become manifest. Usually assumed to be represented in each somatic cell.

A.2. *Non-chromosomal*: all other abnormalities due to the constitution of the gametes, including those which are products of the parental genotypes.

B. *Abnormality due to environmental insult*

B.1. *In post-embryonic life*: abnormalities resulting from interference with a structure after the completion of its primary morphogenesis, but which are not made good by regenerative or other regulative processes.

B.2. *In early development*: abnormalities resulting from the failure of a developing system to regulate after suffering interference.

C. *Experimentally produced artefacts*

Structures which never, or exceedingly rarely, occur in nature. Species or organ chimaerae, induced supernumerary structures, &c.

It must be stressed, however, that an adult organism may owe its normal appearance to regulatory processes (e.g. in cases of incomplete expression of certain genotypes or of embryonic regulation after surgical interference). Here the course of primary morphogenesis has not been normal although its outcome is. We cannot exclude the possibility that the amputation of a normal-looking structure might be followed by the formation of a regenerate showing features of the non-regulated condition. This possibility is perhaps especially important in cases of poor penetrance or minimum expression of genetic defects, but, as will be seen later, has also to be considered in a case where regulation has made good extensive loss of material in early embryonic stages.

Such information as we already have on regeneration after the amputation of abnormal structures, and it includes cases in each of these categories, will be fully discussed in a later paper. All that need be emphasized at this stage is the impossibility of predicting, *a priori*, that a constant pattern of behaviour will emerge within each category. Thus in the cases of A.1, abnormalities, even though we may assume that the genetic constitution of the blastema cells is identical with that of the fertilized egg from which they are descended, it does

not follow that the morphogenesis of a regenerate is sufficiently similar to that of primary embryogenesis for replication of the abnormality to occur. We may well find that the action of certain genes is limited to specific phases of development and that the amputation of a genetically abnormal structure will be followed by the formation of a normal one.

In the previous literature there are two important accounts of work on abnormal amphibian tails. The first, that of Vogt (1931), has received considerable attention. By removing the ectoderm lying laterally to the blastopore of urodele neurulae he obtained larvae in which the ventral fin was missing over part of the length of the tail. When such defective tails were transected in the region of the defect the regenerates subsequently formed lacked a ventral fin. Although he makes only a brief reference to this result it has been taken to show that animals cannot regenerate that which they have never possessed (Huxley & de Beer, 1934), with which view it is, of course, consistent.

Later, Woronzowa & Liosner, of whose work I have only been able to learn from the account in Woronzowa (1949), claimed more surprising results from work with *Rana temporaria*. They produced tadpoles with severe defects of the dorsal part of the tail by removing the dorsal half of the tail-bud of young embryos. Not all their mutilated animals had obviously defective tails, in many cases regulation during urogenesis succeeded in producing an apparently normal tail. Subsequently amputation was performed, the plane of transection being about half-way along those tails which were of normal appearance and anterior to the defective region in the abnormal ones. The majority of all regenerates were of normal appearance, but a substantial proportion showed defects which were generally similar to those appearing in the original tails. Such defective regenerates were formed both in replacement of abnormal tails and of normal ones. A second, and later a third, amputation gave a second and then a final crop of regenerates in which the same general result obtained. Thus even in the group which had normal original tails, and normal first and second regenerates, the third regenerates were in some cases defective.

These results would be remarkable indeed if it could be established that the amputations were actually performed at a level anterior to the defects caused by the primary removal of tail-bud tissue. Unfortunately it is likely that the tails were more extensively defective than would appear from examination of their profile only. Thus the plane of amputation might be chosen to be anterior to a region in which the dorsal fin was missing but still leave a stump in which somites, neural tube, and notochord were defective.

In view of the provisional nature of Vogt's report and the difficulty in assessing Woronzowa & Liosner's results, it was decided to repeat and extend their work. In particular it seemed important to discover whether the regenerative replication of defective tails could take place where the plane of amputation passed through the tail at a level where its cross-section was normal so far as visible structure was concerned.

MATERIALS AND METHODS

Eggs of the axolotl (*Siredon mexicanum*) were used for the urodele experiments and of *R. temporaria* for the anuran ones. Operation procedures were standard. Animals were communally cultured between the infliction of the primary defect and the time of amputation. From then on they were maintained individually in order that each amputate could be compared with its appropriate regenerate.

RESULTS

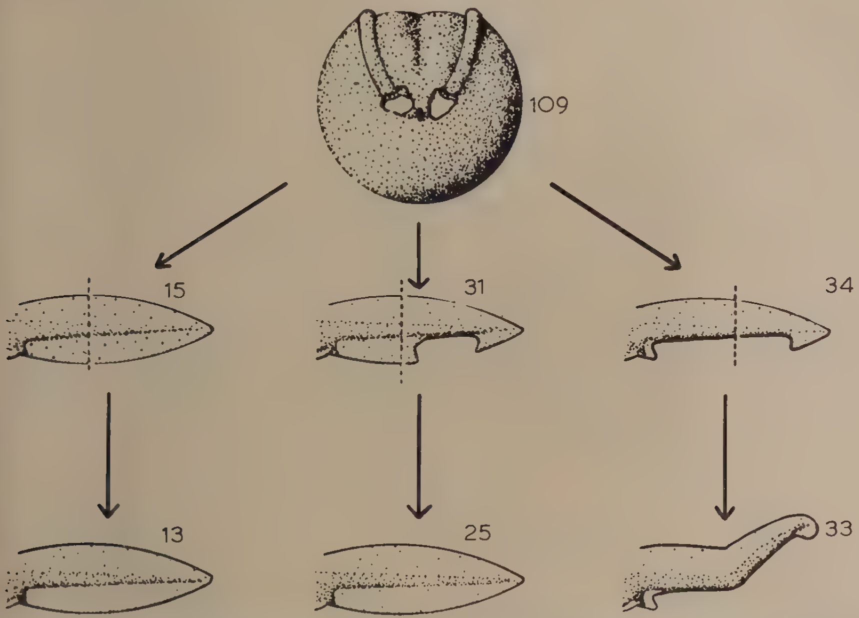
(*a*) *The urodele tail*

Two experiments were performed to test the regenerative properties of defective urodele tails. In the first 109 axolotl neurulae were deprived of their posterior neural folds and some of the adjacent juxta-blastoporal ectoderm (see Text-fig. 1). Most of them subsequently became larvae in which the ventral fin was lacking over a greater or lesser length of the tail, but in some regulation during urogenesis permitted the formation of a normal-looking tail. The ventral fin could be affected from its anterior margin along the anal tube posteriorly to a level just short of the tail-tip. The tail thus never lacked a ventral fin over the last 5 per cent. of its length.

Shortly after the surviving larvae had started to feed all but 12 of them suffered amputation of the tail. In the case of those possessing normal-looking tails the level of transection was approximately half-way between the cloaca and the tail-tip. Defective tails were transected either immediately anterior to the defective region or in the middle of it. Some selection was involved at this point since any larva in which the fin was missing so far anteriorly as the anal tube was automatically placed in the group suffering mid-defect amputation. This was done because it was known that amputation in front of the cloaca is not followed by regeneration in normal larvae. The 12 animals which were left intact had defective tails. They were kept as controls for the stable nature of their defects. In fact, as was expected, they showed no tendency to restore the missing part of their fin. Three months after the other animals had completed regeneration the tails of these 12 too were amputated and they are consequently included in the series as a whole.

The major results of the experiment are shown in Text-fig. 1. Only 71 animals survived until the process of regeneration was complete. Each of the 13 of them which had had normal-looking tails produced a normal regenerate. Each of the 25 regenerates formed after amputating defective tails immediately anterior to their defects was normal in form, though in two cases the ventral fin of the regenerate was remarkable for the complete absence of melanophores. Each of the 33 regenerates formed after the amputation of defective tails at a level where they lacked ventral fin, was abnormal. The abnormality of these regenerates took a constant form, even though the original defects affected differing lengths of the tail. In each case the regenerate as a whole was inclined sharply upwards

at the level of amputation, so that its axis lay at an angle of between 40° and 80° to the axis of the stump. In each case the regenerate lacked a ventral fin posteriorly to a point little short of the tail-tip.

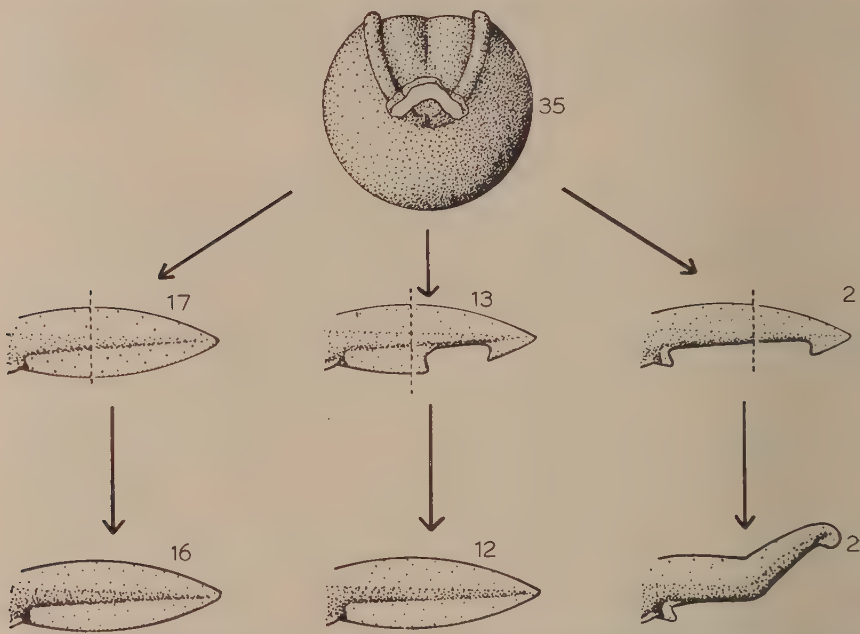


TEXT-FIG. 1. The regeneration of axolotl tails in animals from which the posterior neural folds had earlier been removed, with numbers of animals surviving at each stage.

Histological examination of stumps, regenerates, and amputates revealed no differences between the experimental animals and normal larvae whose tails had been amputated, other than the absence of tail-fin material in the appropriate experimental animals.

The second experiment performed on axolotls differed from the first in one respect only. In 35 neurulae part of the posterior neural plate was removed as well as the presumptive fin material (see Text-fig. 2). This was done in order to produce larvae with tails which had both an externally visible defect in the ventral fin and an internal deficiency of the tail somites. It was to be expected that the somites would be deficient over a region extending farther anteriorly than the fin defect (see, for example, the fate maps of Chuang, 1947). This would make possible the amputation of the tail at a level anterior to the fin defect but within the region of defective musculature. To do this seemed desirable in view of the possibility that the results reported by Woronzowa & Liosner for *Rana* would prove to be explicable in terms of structural deficiency of internal tissues, including somites, at the plane of amputation.

The results, as shown in Text-fig. 2, were precisely parallel to those of the first experiment. Histological examination showed that in many cases the somites were abnormally small in the stump, though in none were they missing altogether.



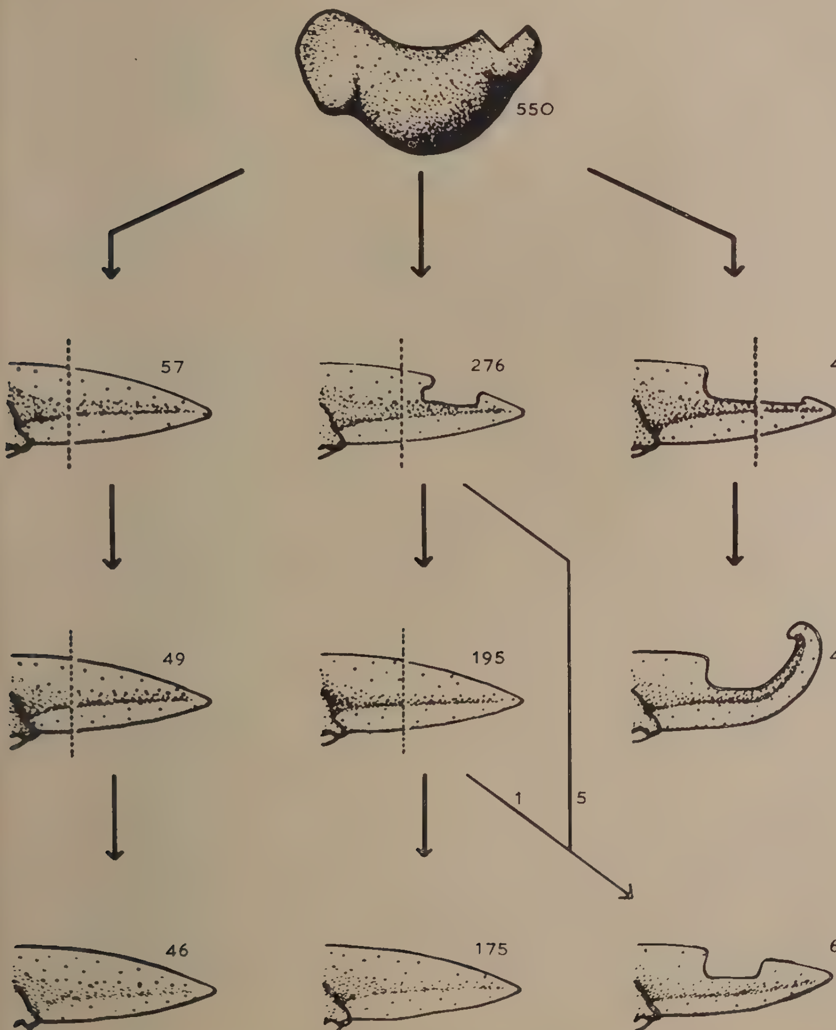
TEXT-FIG. 2. The regeneration of axolotl tails in animals from which the posterior neural folds and part of the medullary plate had earlier been removed, with numbers of animals surviving at each stage.

(*b*) *The anuran tail*

In repetition of the experiment of Woronzowa & Liosner, the dorsal half of the tail-bud was removed from 550 embryos of *R. temporaria*. The great majority of them became tadpoles with defective tails, though some regulated successfully to produce normal-looking ones. The defects were usually gross and affected not only the dorsal tail-fin but also internal tissues. The tails were frequently much distorted, being bent dorsally in the plane of bilateral symmetry.

Of the animals which survived until feeding began, 40 with clearly defined defects were set aside as controls for the stability of their defects. They were kept alive until the onset of metamorphosis but failed to show any tendency to repair their defects spontaneously. A number of other animals were discarded as having tails too malformed for effective amputation. The remainder suffered tail amputation as had the axolotls (see Text-fig. 3). On the completion of the ensuing regeneration most of the tails were re-amputated and a second crop of regenerates produced. By the time these were fully formed the tadpoles were approach-

ing metamorphosis and it was judged impossible to repeat the Russian workers' feat of obtaining three regeneration generations.



TEXT-FIG. 3. The regeneration of tails in *Rana* tadpoles which had earlier lost the dorsal half of their tail-buds. The numbers are of animals surviving to the stages indicated.

The results are shown in Text-fig. 3. The numbers of animals surviving at each stage are shown there. It will be noted that a second amputation was not performed on tails which had been transected in the middle of their defective region.

Clearly the great majority of the experimental animals behaved as did the

axolotls, and this despite the fact that in this case the plane of amputation frequently passed through the tail at a level at which the internal tissues were very abnormal. Indeed, subsequent histological examination showed that effectively normal regenerates were obtained even when the stump tissues were defective in the extreme. Poorly represented notochord, somites reduced to a fraction of their normal cross-sectional area and even a spinal cord reduced to a barely perceptible strand were sufficient to support the formation of a regenerate not markedly inferior to those formed by normal tadpoles.

Six of the regenerates were, however, remarkable in their departure from the general pattern. They all occurred in the series in which tails had been amputated anterior to the defective fin. Five of them were first generation regenerates which also showed a defect in the dorsal fin. The remaining one was formed after the amputation of a normal-looking regenerate.

These six cases are the only ones in which the more unexpected results of Woronzowa & Liosner were repeated. They represent a smaller proportion of a smaller total material than the corresponding cases obtained by these workers. These authors also obtained, it will be remembered, some defective regenerates after the first and second amputations of regulated tails. This phenomenon was not observed in the present experiments.

Histological examination of these six regenerates and of the stumps and amputates of the animals to which they belonged failed to reveal the explanation of the behaviour of the group of five. The cross-section of the stump was, indeed, abnormal in each case, but no more so than in animals which provided normal regenerates. The single abnormal regenerate from the second generation was exceptional in that the spinal cord was not represented at the plane of transection of the first amputation, though it was at the second. In other words the second amputation took place somewhat farther forward than the first. It is difficult to see any connexion between this fact and the aberrant nature of the second regenerate.

It must be recorded that all six abnormal regenerates lacked the dorsal fin over a short region which did not approach the tail-tip at all closely. They were thus quite distinct in appearance from the regenerates formed after mid-defect amputation. On the other hand, though little weight probably attaches to the point, they did not replicate the original defective tails they were replacing in detail.

DISCUSSION

The results of the experiments with axolotls confirm Vogt (1931) in showing that, in contrast to experience with the urodele limb, the structural integrity of the plane of amputation in the tail is necessary for the formation of a normal regenerate—at least so far as the ventral fin is concerned. This is consistent also with the conclusions drawn by Holtzer *et al*. (1955) and Holtzer (1956) from their experiments. They recognize that the cartilages and connective tissue of a uro-

dele tail regenerate are formed by blastema cells analogous to those from which a limb is regenerated, but find that muscle and spinal cord spring more directly from their equivalents in the stump. The ventral fin may now, with some confidence, be added to the latter two.

We must admit that the material described provides no evidence for a general dependence of regeneration upon primary urogenesis, except in so far as this is necessary to provide a plane of amputation of normal cross-section. Those authors who have seen in Vogt's results a demonstration that organisms cannot regenerate what they have never possessed are not really justified. It is, of course, true that a larva which lacked the whole of its ventral fin could not be given one by amputating the tail, but this is because the fin normally extends anteriorly to the cloaca and amputation in front of this point is not followed by regeneration at all. It is also true that one can devise an experiment in which the regenerate mimics the original tail in point of ventral fin deficiency. But this can only be done where the original deficiency extends posteriorly to a level just short of the tail-tip. The correspondence is in this case fortuitous, however, since what determines whether the regenerate should have a complete ventral fin or none at all is the presence or absence of fin at the plane of amputation. The same animal could be made to regenerate a normal tail if the level of amputation were shifted forwards into a region with the fin intact.

However, it is important to recognize the possibility, in principle, that the process by which an abnormal tail is replaced by a normal one may differ from normal regeneration although its outcome does not. That is to say, it remains possible that where primary urogenesis is abnormal, regeneration to form a normal tail calls upon regulative processes that are not usually involved. This possibility would have to be taken seriously if, in a proportion of cases, regulation were apparently incompletely effective and the regenerate showed some sign of abnormality.

This, at first sight, might appear to be so in the experiments of Woronzowa & Liosner and in the experiments on *Rana* reported above which repeat them. However, the assessment of these results is, unfortunately, not a simple matter. In the first place it must be admitted that the abnormal regenerates which do not conform to the general pattern are so few that they might be regarded as the products of contingent events. Indeed, Woronzowa states that she obtained a higher proportion of abnormal regenerates among animals whose culture conditions were poor than among those which were really healthy. On the other hand, I have not obtained similar abnormalities in cultures of normal tadpoles whose tails were amputated. Far more important is the fact that in the experiments on *Rana* there was no question of the cross-section of the tail at the level of amputation being normal. It was in all cases very clearly defective. It is therefore simpler to view the occasional abnormal regenerate as the product of failure to regulate in a situation rendered abnormal not by the more remote events of unusual urogenesis but by the immediate consequences of a defective stump.

We may conclude that amphibian tails bearing witness to defects incurred during the mosaic phase of development do not necessarily produce abnormal regenerates after transection in front of the defective part, and that there is no firm evidence for regarding the regeneration that takes place as differing in any respect from the normal.

SUMMARY

1. The bilateral removal of the posterior neural folds of axolotl neurulae may lead to the development of larvae in which a greater or lesser length of the ventral fin of the tail between the cloaca and a point just short of the tail-tip is missing. In other cases regulation permits the development of a normal-looking tail.

2. The later amputation of such 'normal' tails, or of defective ones where the plane of amputation lies anterior to the defective region, is followed by the formation of normal regenerates.

3. The amputation of defective tails where the plane of amputation passes through a region lacking ventral fin is followed by the formation of a regenerate lacking ventral fin as far posteriorly as a point just short of the tail-tip.

4. The presence or absence of a ventral tail-fin in the regenerate depends upon its presence or absence in the plane of amputation, and is not otherwise related to the degree of abnormality of primary urogenesis.

5. Tails which have defective ventral fins and poorly developed somitic musculature (as a consequence of simultaneous removal of posterior neural folds and part of the posterior medullary plate) behave in regeneration as do those lacking ventral fin only, even where the plane of amputation passes through a region with deficient somites.

6. The removal of the dorsal half of the tail-bud of embryos of *R. temporaria* may also be followed by almost normal urogenesis as a consequence of regulation. In most cases, however, the resulting tails are extensively defective lacking a region of the dorsal fin, parts of the spinal cord, and having poorly developed somitic musculature.

7. In nearly every case the amputation of these tails was followed by the formation of a regenerate that was normal if the original tail was 'normal' or if the plane of amputation lay anterior to the region of the tail which lacked a dorsal fin. Where the plane of amputation passed through a region lacking dorsal fin the regenerate was formed without one.

8. In a very small number of cases exceptional and defective regenerates were formed, either after a first or a subsequent amputation, where normal ones were to be expected. The occurrence and significance of these exceptions are discussed.

9. It is concluded that the successful completion of normal primary urogenesis in amphibians is not a prerequisite of the formation of a normal

regenerate. It remains possible that the formation of normal regenerates from abnormal tails involves regulative properties in the blastema.

10. The need for representation of tail-fin at the plane of amputation if it is to be present in the regenerate is further confirmation of the departure of amphibian tail regeneration from the pattern established for the urodele limb.

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Morphogenetic Activities during Planarian Regeneration as influenced by Triethylene Melamine

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WITH TWO PLATES

INTRODUCTION

THE extensive investigations undertaken during the last few years to find chemotherapeutic substances for treatment of neoplastic diseases have been of great importance to experimental morphology in the study of the causal relations between morphogenetic processes. Most of these compounds have a profound influence on proliferating tissue. The assay systems have of course been especially malignant tumours of mice or rats. The morphogenetic potentialities of neoplasms, however, are rather restricted, mainly being limited to proliferation. Proliferating systems with a wider scope of potentialities are embryos and regenerating organisms. Anti-neoplastic compounds of promise have gradually been subjected to a closer biological analysis using these systems, e.g. 6-mercaptopurine (Bieber *et al.*, 1954), demecolcin (Steinmann, 1954), nitrogen mustards (Bodenstein, 1954), urethane (McMillan & Battle, 1954), triethylene melamine (Töndury, 1955), and azaserine (Dagg & Karnofsky, 1955). The results obtained have as a rule been of general interest, both for the evaluation of their application through the knowledge of their side-effects and mode of action, and for the insight into morphogenetic processes gained by an analysis of the derangements in development encountered. By a comparison of the information obtained about the action on neoplasms, on the one hand, and on embryonic or regenerating organisms, on the other, it should be possible to add to the knowledge of neoplastic growth and to visualize neoplasia as a morphogenetic process, though a deviant one.

At the time when the present study was initiated one of the most successful anti-neoplastic substances discovered was triethylene melamine (TEM) (Hendry *et al.*, 1951). In human medicine it has been especially effective in alleviating lymphatic leukemia. It was thought worth while to study its actions on regenerating planarian worms, since these animals quickly regenerate lost parts. The

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species used for this study, *Planaria vitta*, will completely regenerate a head in less than 2 weeks after decapitation.

The present paper describes the normal morphogenetic processes of head regeneration in *P. vitta* and their modification under the influence of TEM. The partial processes considered are mitosis, cell migration, induction, differentiation, inhibition, and the two modes of regeneration, epimorphosis and morphallaxis. The closely related phenomenon of asexual reproduction (fission) is also touched upon, and some histochemical and biochemical observations are considered.

MATERIAL AND METHODS

The animals

The unpigmented *P. vitta* was mainly employed. This triclad flatworm reproduces asexually by fission which in most cases takes place caudal to the pharynx. It is provided with two eye-spots situated dorsal to the middle of each of the two brain ganglia. The pigmented *Euplanaria polychroa* was used for the amino acid determinations, for which it was preferable because of its larger size. To get as pure a material as possible, cloned animals were used throughout. During the experiments all the animals were kept in darkness in Petri dishes with 50 ml. tap-water in a thermostat at 20° C. No food was allowed during the experiments, and as a rule the animals had been starved for some time before decapitation, partly because starvation decreases the rate of fission, partly because it stabilizes the internal milieu. The food normally given consisted of enchytraeids.

Only head regeneration was studied. Regenerating planarians were obtained by decapitation, a transverse cut being made just behind the eye-spots, removing the brain ganglia too. Care was taken that all the animals were cut at a uniform distance behind the eyes. As described by Brøndsted (1955), planarians possess a time-graded regeneration field specific for each species. The rate of regeneration is dependent on the position of the cut in this field. Before decapitation the planarians were anaesthetized with nicotine sulphate 1:10,000. As a measure of the regeneration rate, the first day upon which two eye-spots could be discerned with the binocular microscope at a standard illumination and magnification ($\times$ 20) was noted.

The substance

Triethylene melamine (TEM) is 2:4:6-tris-ethylene-imino-1:3:5-triazine. This compound has radiomimetic actions, and it is very similar to the mustards in its biological effects. It is easily dissolved in water. For the experiments it was dissolved in tap-water, and new solutions were made every day. TEM in aqueous solution is not stable. Plummer *et al.* (1952) reported a decrease in activity of 6 per cent. each 24 hours at room temperature. Even crystalline TEM is not quite stable. If the same batch is used higher concentrations have to be employed as time goes on. To test the stability of the substance spectrophotometric

measurements were made over a period of $1\frac{1}{2}$ years in the range 210–400 mμ. The maximum was unchanged about 220 mμ, and the shape of the curves identical. Most of the experiments reported here were made during a period such that it was not necessary to raise the concentration of the TEM-solution in order to get identical biological responses.

Histological technique

The planarians were fixed in buffered formalin (4 per cent., pH 7·0), Carnoy, acetic alcohol 1:4, or Helly. This last fixative produces by far the best histological picture, especially as regards cytoplasmic structures. It usually involves, however, considerable curling of the animals. Carnoy or acetic alcohol, on the other hand, penetrate so rapidly that it is possible to fix the animals outstretched without appreciable curling. They fix nuclei and neoblasts well, and they were used routinely. Fixation is quick because of the small size of the animals (only *P. vitta* was studied histologically). Twenty to thirty minutes in Carnoy or acetic alcohol is enough, and 1 hour in Helly. In formalin the animals were fixed for 24 hours. After washing, dehydration, and clearing in methylbenzoate and xylene, they were embedded for 20 minutes in paraffin and cut sagittally, in a few cases frontally, at 5–6 μ. Some animals were stained routinely in iron trioxyhematein-eosin. This staining method, however, does not produce as clear a picture of the neoblasts as desired. These cells are heavily loaded with RNA, and to get a clear picture of them histochemical methods for nucleic acids were employed. In a few series the gallocyanin-chromalum method of Einarson (1951) was used after fixation with Carnoy. The animals were stained for 48 hours at a pH of 1·64. Some of the slides were counterstained with eosin. In some cases a pretreatment with ribonuclease (Sigma) was carried out. One series was stained with methyl green pyronin (Pearse, 1953, p. 427) for about 18 hours. Both stains were obtained from Merck. The procedure was modified in that the concentration of methyl green was doubled. Of several pyronins employed that of Merck produced the clearest picture of the neoblasts.

An easy and reproducible staining method adopted for the neoblasts is the Giemsa stain. After fixation in acetic alcohol and sectioning, the slides were stained with Giemsa diluted 1:15 with phosphate buffer at pH 6·9 (N. O. Lindh, personal communication) for 18 hours. After staining, there followed a short rinse in buffered distilled water (10 seconds), and differentiation for 2 minutes each in 70 per cent. ethanol and two changes of absolute ethanol. After clearing in two changes of xylene the slides were mounted in DPX (Edward Gurr). It is essential that the mounting medium is neutral. If not, the stain is unstable. In a few cases where more cellular details were desired, Helly-fixed animals were stained with Heidenhain's azan.

The PAS reaction of Hotchkiss was employed on formalin-fixed planarians. A reducing rinse was used after the periodate oxidation, but to overcome a possible blockage of the exposed aldehyde groups, a 30-minute immersion in Schiff's

reagent was employed. The slides were counterstained with iron trioxyhematein. Controls not exposed to oxidation were stained, but were completely PAS-negative.

Counting of mitoses

A squash technique was adopted. The animals were pretreated in a mixture of 1 part of 1 N hydrochloric acid to 10 parts of a 2 per cent. orcein (Merck) solution in 45 per cent. acetic acid. After treatment for 10 minutes the animals were transferred to a drop of orcein-acetic acid on a slide. After cutting, the animals were squashed. The preparations were made permanent by freezing with carbon dioxide, separation of coverslip and slide with a razor, dehydration with absolute alcohol, clearing in xylene, and embedding in Canada balsam. All mitoses were counted with the exception of prophases which are difficult to define in this species because of the dense chromatin in the interphase nuclei. The number of interphase nuclei was determined afterwards by sampling, every 25th field being counted. The counting was done at a magnification of 680, using a dry objective ($\times$ 40).

Respiration measurements and total nitrogen determination

The measurement of oxygen uptake was made with the Cartesian diver technique of Holter & Linderstrøm-Lang as modified by Zeuthen (1950). The technique is described in a previous paper (Pedersen, 1956). The total nitrogen determination was made by direct nesslerization. A Beckman DU spectrophotometer was employed instead of, as previously, the Eel colorimeter, the measurements being made at 500 mμ.

The determination of free amino acids

For this purpose ascending paper partition chromatography was employed. The first run was made in a mixture of secondary butanol (300 ml.), formic acid 99 per cent. (52·5 ml.), and redistilled water (47·5 ml.). Whatman paper No. 1 (30 cm. by 30 cm.) was used. It was run for 12 hours in the first solvent. After drying for 3 hours at a constant temperature of 23° the chromatograms were transferred to a solution of phenol (Riedel de Haen) in redistilled water, 80 per cent., stabilized with 0·04 per cent. 8-oxyquinolin. A run of 15 hours was employed. After drying at room temperature the chromatograms were sprayed with a mixture of 50 ml. 96 per cent. ethanol, 15 ml. glacial acetic acid, 2 ml. collidine, and 100 mg. ninhydrin. They were then developed in an oven at 60° C. for 20 minutes. The localization of the amino acids had previously been determined by running individual amino acids and mixtures of them.

RESULTS

1. *Description of normal regeneration in* P. vitta

In planarians both epimorphic and morphallactic processes of regeneration are drawn upon, but at different times. Epimorphosis precedes morphallaxis,

though there is no sharp transition between the two. Epimorphic processes are the better known. They include a regressive phase and a progressive constructive phase. The latter can be further divided into three processes: (1) initiation, activation, and formation of a blastema; (2) growth of the blastema; (3) differentiation of the blastema. These processes are not, of course, sharply separated, but in, for instance, amphibian tail-regeneration (*Xenopus* larvae) they can be differentially inhibited (Lehmann & Bretcher, 1952). Important for the regulation of regeneration are the limiting processes which inhibit the positive regenerative processes, once they are initiated. In planarian regeneration they have been most thoroughly considered by Brøndsted (1956), who has been able to measure the time involved in the spread of the inhibiting forces.

The epimorphic part of regeneration in *P. vitta* may arbitrarily be defined as lasting until the appearance of the two eye-spots, i.e. about 10 days. Morphallaxis has by then already begun to play its part, and through imperceptible steps it remodels the animal to a harmonious shape.

Regeneration proper is the result of many partial cellular processes which are more or less independent of each other. Just after decapitation a strong contraction of the wound follows, and in a few hours epidermal activity begins. On the 3rd day epidermal closure is complete. During the early days the epidermis is rather thin, consisting of squamous cells. On the 3rd and 4th days rhabdites are again accumulating in the epidermal cells of the blastema. Wounding, through as yet unknown processes, activates migration of totipotent regeneration cells, the neoblasts, found in the parenchyma. This migration is already evident on the day of decapitation and is very active for the next few days. Mitosis of neoblasts is evident the 1st day after decapitation. It reaches a maximum on the 3rd and 4th days. Mitoses may be found everywhere in the body, but as a rule they gradually become concentrated in the area adjacent to the distal part of the blastema after an initial, more uniform, distribution.

The growth of the blastema itself is easy to follow, because the head region and the blastema region are transparent. The rest of the body, however, is opaque owing to the presence of the diverticula of the digestive tract. A little streak of blastematous tissue is visible under the dissecting microscope on the 3rd day after decapitation. Serial sections show, however, that already on the day after decapitation a small blastema has formed. An increase in size is evident about the 4th–5th day (Plate 2, fig. E), and during the 5th–7th days growth of the blastema is most rapid. Further growth goes on after this, but at a reduced rate.

Differentiation of the two brain ganglia begins early. It may be observed on the 4th day after decapitation, and on the 5th and 6th days, the ganglia can be discerned with certainty. The eyes are formed as a result of induction from these ganglia (Wolff, 1953; Lender, 1952). They may be seen as early as a week after decapitation in this species.

As previously mentioned, the time taken for the eye-spots to appear is used as

measure of the regeneration rate in these experiments, so a few remarks on its eproducibility may be appropriate. Graphs are constructed, using days after lecapitation as abscissae and percentage of regenerated animals (i.e. animals vith two eye-spots) as ordinates. The day by which 50 per cent. of the animals ave regenerated is called the RR_{50}. This measure has been determined for 16 ontrol experiments carried out over $2\frac{1}{2}$ years, each experiment comprising 20 nimals, starved for about a month and kept at 20° C. in the dark. The mean nd its standard error for these 16 groups is $9{\cdot}4 \pm 0{\cdot}4$, so it will be realized that he variation is rather slight. For animals not starved before decapitation a value f 8·8 has been found in an experiment comprising 20 planarians. The retardaion in the regeneration rate due to one month's starvation is accordingly only bout 7 per cent., obviously not significant.

. The action of TEM on the regeneration rate

As was to be expected, TEM strongly influences the rate of regeneration. In wo experiments on 160 planarians, 20 in each of 8 groups, 2 groups being conrols, the effect of continuous exposure to TEM was determined. In the first xperiment, 50, 5, and 0·5 mg. TEM per litre caused a complete suppression of egeneration. None of the animals developed two eyes. In another experiment, ısing 1, 0·1, and 0·01 mg. TEM per litre, 1 mg./l. involved total suppression,)·1 mg./l. a strong retardation, while 0·01 mg./l. was without effect.

A discontinuous exposure to TEM is advantageous for a closer study of its ction. An exposure to 15 mg. TEM per litre for 45 minutes just after decapitaion produces a clear retardation with no mortality. Exposure to 50 mg. TEM er litre for 1–3 hours, followed by decapitation 2–3 hours or 24 hours after, esults in a long-lasting retardation, and this mode of application has been specially employed in studies on morphallaxis. This treatment does not kill the nimals either.

3. Toxicity of TEM

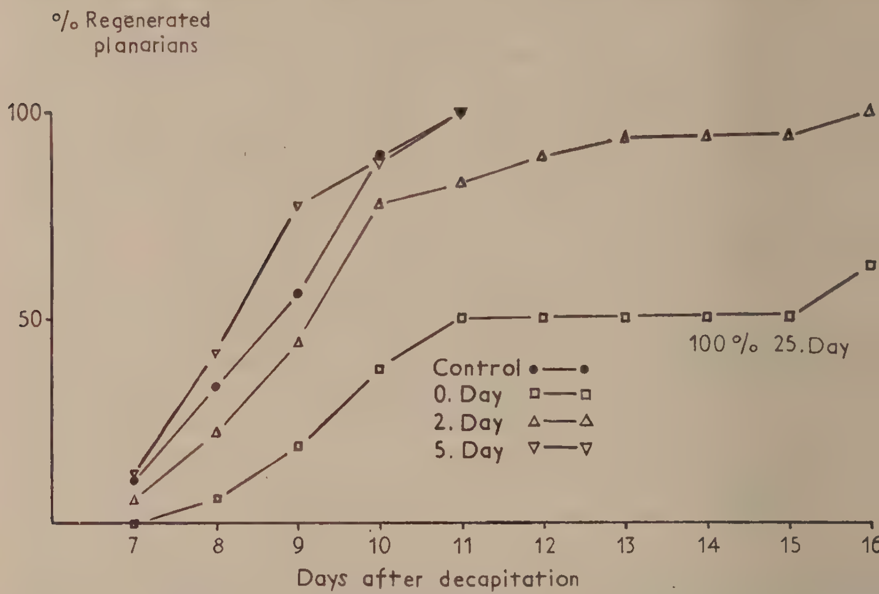
The toxicology of TEM has been studied in mammals by Hendry *et al.* (1951). In planarians, as in mammals, it is highly toxic. In groups of 20 animals each treated continuously with concentrations of 50, 5, and 0·5 mg./l., all animals were dead on the 13th, 20th, and 43rd day respectively. Some animals were transferred to tap-water after TEM administration for 16 days. There was no recovery.

TEM affects especially, though not exclusively, proliferating tissues. It is of interest, therefore, that exposure to TEM at 50 mg./l. gave no indication of a greater mortality in decapitated than in intact animals.

4. The time of action of TEM

One of the crucial problems as to the action of TEM is the correlation between the time of administration and the effect obtained. This was studied in several experiments. In one experiment 3 groups of unstarved planarians, each consisting

of 20 animals, were exposed for 45 minutes to TEM, 15 mg./l. (Text-fig. 1). The first group was exposed just after decapitation (day 0), the next on the 2nd day, and the last on the 5th day after decapitation. The animals were carefully rinsed after the exposure. An obvious effect was only observed in the group



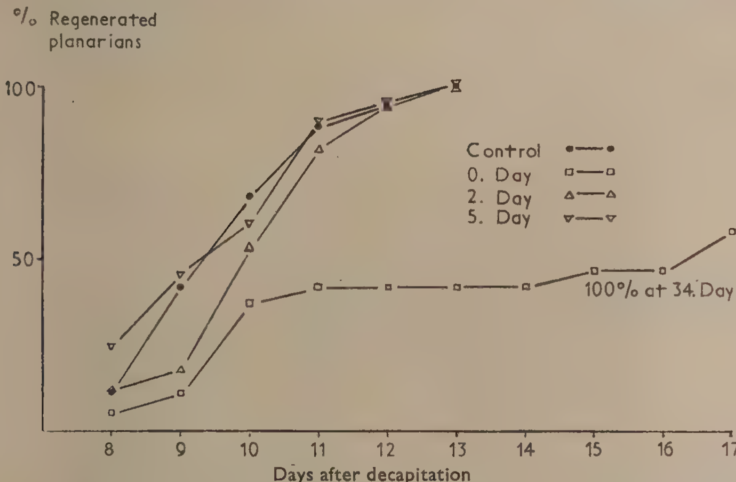
TEXT-FIG. 1. Regeneration rate of planarians treated with 15 mg. TEM per litre for 45 minutes at different times after decapitation. Not starved before decapitation.

exposed on the day of decapitation. The curve showing the regeneration rate after exposure on the 2nd day is normal to the 10th–11th days. The RR_{50} is within the normal range. The rather abnormal course after the 11th day is probably due to the inclusion of a few animals especially sensitive to TEM. A rather great scatter in regeneration experiments with antimitotic substances has also been encountered by Lehmann & Bretcher (1952) and Wilhelmi & Steinmann (1950). Its physiological basis is not known with certainty, but it may be partly due to differences in mitotic activity at the time of application of the substances.

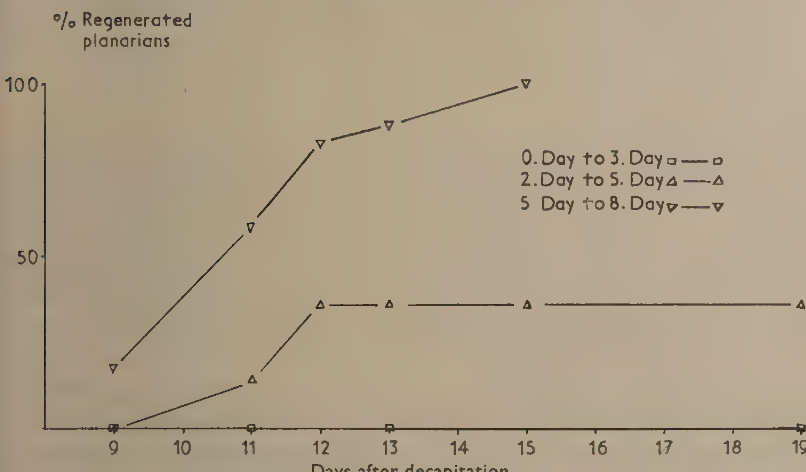
The same kind of experiment was carried out with 80 planarians starved for one month before decapitation (Text-fig. 2). It is evident that starvation does not alter the time relations, but the effect of TEM is, if anything, somewhat more pronounced. In this experiment an abnormal scatter in the group treated on the 2nd day, as with the unstarved planarians, was not observed.

In another experiment the effect of a more drastic treatment with TEM was studied. Here the animals were treated with TEM 5 mg./l. for 3 days in the early, middle, and late phases of regeneration. Each group consisted of 16 planarians

Text-fig. 3). The group treated for the first 3 days of regeneration showed complete suppression of regeneration, the group treated in the middle phase a severe retardation. About 60 per cent. of the animals here died without regenerating



TEXT-FIG. 2. Regeneration rate of planarians treated with 15 mg. TEM per litre for 45 minutes at different times after decapitation. Starved for 1 month before decapitation.



TEXT-FIG. 3. Regeneration rate of decapitated planarians treated for 3 days in the early, middle, and late phases of regeneration with 5 mg. TEM per litre.

two eyes. The group treated in the late phase of regeneration (5th–8th days) is a little retarded. Compared with normal regenerating animals its RR_{50} is delayed by about 1 day. The shape of the curve is quite normal.

There is also an effect of TEM when planarians are treated before decapitation. In one experiment TEM at a concentration of 50 mg./l. was applied for 2 hours. Decapitation was performed in one group 2 hours, in another group 24 hours, afterwards. Complete suppression of regeneration for some months was the result. There were no signs of any lethal action. The two groups did not differ in the ensuing regeneration.

This last experiment also bears on the role played by the penetration of the substance. As previously mentioned, wound-closure is very rapid in planarians. The epidermis is healed in a day or two and provisional closure is secured even earlier through contraction of the wound and the rapid extension of epidermal cells. The experiment just described clearly shows that penetration occurs rapidly in intact planarians. In another experiment planarians on the 5th day of regeneration had a bit of their tails cut off and were exposed to TEM at 15 mg./l. for 45 minutes. There was no effect of the exposure. It should be mentioned that a second wound on a planarian already regenerating a head does not decrease the head-regeneration rate (Brøndsted, 1956). It is probable that an influence of permeability can be ruled out, and that the time relations of TEM action are due to interaction with the particular processes going on at that time in the regenerating organism. A phase specificity is thus found.

The outcome of these experiments is, therefore, that TEM acts at a specific time in regeneration: in the first phase only after rather mild treatment, and in the first and middle phases after drastic treatment. The time of action of TEM is centred particularly around the period of blastema establishment. The main cellular processes then involved are migration of neoblasts to the wound and their mitosis.

5. *The influence of TEM on mitotic activity and migration*

In one experiment 50 planarians starved for about a month were decapitated. The animals were selected so as to possess a pharynx situated as caudal as possible. This morphological state is correlated with a morphogenetic one, the animals having undergone fission lately. This selection should ensure that mitotic activity connected with asexual reproduction is not a complicating factor. Half of the animals were exposed continuously to TEM at a concentration of 5 mg./l. Every day for the following 9 days two animals from each group were squashed as previously described. Squashing was performed at the same time every day to avoid the influence of a possible diurnal rhythm. In another planarian species, *Polycelis nigra*, such a rhythm has, however, not been found (Verhoef, 1946). The result of the mitosis counting can be found in Table 1. The mitotic activity in intact animals is below 1 per 1,000. Decapitation involves a rise in activity, which is most prominent on the 3rd and 4th days. During the following days, the differentiation period, it again decreases. The peak in mitotic activity coincides with the first visible increase in size of the blastema, and it precedes the period of maximum blastema growth rate (5th–7th days). This

lagging of growth behind mitotic activity is explained by the fact that the majority of mitoses proceed caudal to the blastema and at its border. Growth of the blastema follows the migration of proliferated neoblasts. The animals treated with TEM exhibit a severe decrease in mitotic activity, but it is not quite abolished even on the 5th day (5 metaphases found). There was a considerable preponderance of metaphases as compared with ana- plus telophases in the treated animals, the ratio rising with the time of exposure to TEM.

TABLE 1

Mitotic rate during regeneration. Experimental animals treated continuously with 5 mg. TEM per litre

Day after decapitation	*Controls not treated*			*TEM-treated*		
	Mitoses	*Nuclei*	*Per 1,000*	*Mitoses*	*Nuclei*	*Per 1,000*
Intact	60	3,012	0·79	..	..	..
,,	128	5,945	0·86	..	..	..
1st day	238	6,011	1·58	44	3,379	0·52
,,	..	..	..	66	4,485	0·59
2nd day	228	5,847	1·56	47	6,072	0·31
,,	152	4,606	1·32	..	..	..
3rd day	241	3,978	2·42	22	6,005	0·15
,,	411	3,494	4·71	..	..	..
4th day	413	3,721	4·44	8	4,807	0·07
,,	322	5,243	2·46	..	..	..
5th day	327	7,619	1·72	5	3,897	0·05
,,	178	4,407	1·62	..	..	..
6th day	148	4,531	1·31	..	..	..
7th day	138	5,045	1·09	..	..	..

Another experiment involved a short-term treatment with TEM, 2 hours at a concentration of 50 mg./l. The next day decapitation was performed. After 5 days two animals from each group were squashed and examined, but no counts were made. Only a few mitoses (both metaphases and anaphases) were observed in the treated animals, while the controls exhibited vigorous mitotic activity.

These experiments indicate that TEM strongly affects the recruiting of material for the blastema through mitosis of neoblasts.

The interesting morphogenetic consequences of this suppression will be dealt with below.

In this connexion the effects of TEM on cell migration may be mentioned. Evidence of migration can only be obtained indirectly. Experimental proof of its existence and fundamental significance in planarian regeneration has been given by Dubois (1949). In the present study migration was followed indirectly through examination of serial sections of animals during the whole period of regeneration (Plate 2, fig. F). Evidence of migration was always observed, even at the highest concentration used, 50 mg./l. Tissue-culture studies on TEM (Plummer *et al.*, 1952) show that migration of chick-heart fibroblasts is little

affected and never completely checked, even in cases where mitotic activity had been completely abolished.

6. *Epimorphosis and morphallaxis in relation to TEM treatment*

It has previously been mentioned that in normal planarian regeneration, epimorphosis is responsible for the first and most obvious phase. A blastema develops and differentiates into a new head with brain ganglia and eyes. Later on morphallaxis plays its part in remodelling the animal to harmonious shape. TEM influences these two phases in a very characteristic way.

The presence of a blastema is, of course, indispensable for epimorphosis. When planarians are treated with TEM the size of the blastemata are no different from those of regenerating controls up to the 5th or 6th day. But from this time on a strong hypoplasia in the treated animals is observed, its extent being somewhat dependent on the concentration of TEM (Plate 2, figs. E and F). In the experiments where the TEM treatment was intense (e.g. 50 mg./l. or 5 mg./l. continuously) the hypoplasia was, in addition, followed by a pronounced regression of the blastema.

In the animals displaying the most hypoplastic blastemata a curious phenomenon was observed. Some animals had been treated with 50 mg./l. TEM for 3 hours, rinsed and decapitated 24 hours later. About the 10th day after decapitation two hypoplastic brain ganglia appeared between the branches of the digestive tract in the opaque part, that is, in the old part of the animal. The blastema was only a small streak. After some time first one and then the second eye appeared, immediately dorsal to the brain ganglia as usual. After a further month, a little more than 2 months after decapitation, all animals were quite normal with two eyes in a translucent head region of normal shape and size.

This phenomenon was studied more closely in an experiment with 20 planarians decapitated 24 hours after treatment with TEM at 50 mg./l. for 2 hours. The same results were obtained, no epimorphic regeneration occurring in the animals, which had almost no blastemata. The time of appearance of brain ganglia and eyes showed great individual differences. Regeneration of two eyes took place over a period of $1\frac{1}{2}$ months; and 3 months after decapitation all planarians were quite normal with a translucent head region. The phenomenon may be interpreted as a morphallactic regeneration displaying itself as a consequence of the inhibition of epimorphosis. The morphallaxis occurs in two phases. First a preliminary morphallaxis with differentiation of brain and eyes in the old part of the decapitated animal, followed gradually and very slowly by the re-establishment of a harmonious animal.

The cellular processes have not yet been followed in detail. In one case two animals were fixed in Helly 25 days after decapitation. Both of them showed morphallaxis macroscopically, two brain ganglia connected with a transverse commissure appearing in the old part of the animal. One animal had, moreover, regenerated two eye-spots. Microscopical examination corroborated the obser-

vation. In both cases two very elongate hypoplastic ganglia were found. They were cranially displaced, and ventral to them gut diverticula were found. Scattered neoblasts were present, and some were engaged in further regional differentiation of the brain ganglia, the process going on cranial to these (Plate 1, fig. A). Neoblasts were also engaged in differentiation of muscle. A blastema was not present. The region cranial to the brain was considerably less cellular than usual and the parenchyma looser.

The results considered above are the outcome of experiments in which rather drastic treatment with TEM was used. If a more moderate treatment is employed, e.g. 15 mg./l. for 45 minutes just after decapitation, morphallaxis is not always the sole regenerative response. In some cases, eyes have differentiated in a hypoplastic blastema. In the majority of animals, however, morphallaxis is encountered, but the secondary morphallaxis with readjustment to normal shape follows considerably more rapidly than in the animals treated more intensely.

7. *Differentiation and induction in relation to TEM treatment*

Only two systems were examined, the differentiation of brain and of eye-spots. In one experiment decapitated planarians were exposed to TEM 5 mg./l. continuously. Two eyes are never regenerated after such treatment, but occasionally one (see next section). Nevertheless, hypoplastic brain ganglia had differentiated, though at a slower rate than the controls (Plate 1, fig. B). There was generally a delay of a few days. The development of the ganglia was rather poor and their regional differentiation was much restricted and as a rule lacking. If the treatment was milder, regional differentiation might progress, but at a decreased rate. In many cases one of the brain ganglia exceeded the other in size. When treated with toxic concentrations a regression of the newly formed hypoplastic brain followed. In the experiments involving a very high concentration (50 mg./l.) brain formation was completely inhibited. From these observations it can be concluded that TEM (except in immediately lethal concentrations) is not an inhibitor of brain differentiation. Differentiation proceeds, but at a reduced rate, as a consequence of the poor availability of neoblasts.

Differentiation of the eye-spots is the result of an induction emanating from the brain ganglia through diffusible substances (Lender, 1952; Wolff, 1953). In regenerating planarians treated with TEM there is, as a rule, a pronounced time-lag between the differentiation of a brain and the appearance of the eye-spots. The delay is especially marked in the experiments involving exposure to 50 mg./l. TEM for 2 hours on the day before decapitation. This experiment may be used as a demonstration of the fact that TEM does not interfere with induction *per se*. The inductive process takes place from several weeks to 3 months after treatment with TEM has been concluded, and at this time not a trace of TEM would have been left. In addition, treatment with TEM at the time of induction (Text-fig. 3) was without action. The reason for the delay between the appearance of the brain and the induction of the eye-spots is

certainly the hypoplasia of the brain. Because of this, production of the inducing substances may not reach the critical level for induction to take place. This delay, too, is therefore due to scarcity of material for regeneration.

8. *The one-eye phenomenon*

In Text-figs. 1 and 2 the graphs showing the regeneration rate after treatment with TEM on the day of decapitation exhibit plateaus for several days (day 11 to day 15). This of course means that during this time no animals with two eyes were observed. Nevertheless, they had as a rule regenerated one lateral eye growing in size. It should be emphasized that this condition is not related to cyclopia. Neither was the range of eye defects described by Child (1941, p. 178) encountered. The single eyes noted in these experiments were always located distinctly laterally. The single lateral eye was present for a time, depending on the mode of treatment and concentration of TEM. In some cases where lethal concentrations were employed the animals died without the appearance of the other eye. Other cases remained more than a month without any signs of eye formation on the other side. The phenomenon was observed in all experiments except those with a continuous administration of TEM in a concentration of 50 mg./l. Macroscopically it was frequently noted, and confirmed microscopically, that the brain ganglion situated just ventral to the single eye-spot was larger than the corresponding ganglion on the other side. It was noted, moreover, that both ganglia were hypoplastic relative to the controls. The size of the regenerated eye-spot depended on the concentration of TEM employed. In experiments with continuous administration of TEM (e.g. 5 mg./l.), where regeneration of two eyes never occurred, the single eyes were as a rule smaller than in the controls. In experiments with interrupted treatment and subsequent, though delayed, regeneration of two eyes, the single eye most frequently grew to the normal size before the other eye regenerated. Often there was a further time-lag until the formation of the corresponding eye. Some figures may be cited to indicate the frequency of the phenomenon. Animals (20 in each group) exposed to 1 and 5 mg./l. continuously produced 20 per cent. and 50 per cent. of animals with a single lateral eye. In both cases all animals died before regenerating the other eye. In the experiment, the result of which is given in Text-fig. 3, 6 of the remaining 9 planarians had one lateral eye. Here also the animals died without regenerating the other eye.

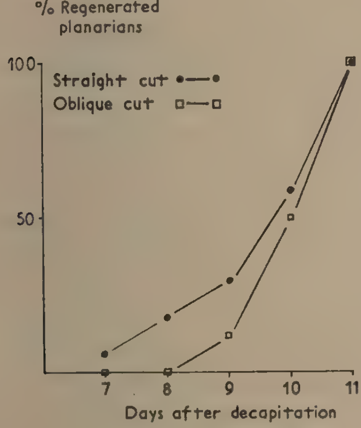
In the animals of Text-fig. 2 all had regenerated one eye on the 17th day; not until the 34th day after decapitation had all animals regenerated two eyes and even on the 10th day 83 per cent. of the remaining animals had regenerated only one eye.

Some experiments employing another substance with a comparable depressive action on regeneration, but with another point of attack, may be discussed. BAL (2 : 3-dimercaptopropanol) was administered for 12 days after decapitation (20 animals in each group) in concentrations of 10, 1, and 0·1 mg./l. In concentra-

tions of 10 and 1 mg./l. a complete blocking of regeneration was noted up to the 12th day, while the group treated with 0·1 mg./l. were strongly retarded. After transfer to tap-water the animals recovered and regeneration ensued, quickly after the treatment with 0·1 and 1 mg./l. and very slowly after 10 mg./l. (the animals were observed for 3 months). Hence no phenomenon comparable to that described above was observed. There was a normal time-lag between the appearance of one lateral eye and of the contra-lateral one. Furthermore, in the group treated with the highest concentration many malformations were observed, e.g. cyclopia and formation of supernumerary eyes. Malformations of this kind were not seen in animals treated with TEM. As will be discussed below, all the anomalies observed after TEM treatment may be referred to its primary cellular action—destruction of the neoblasts and consequent interference with mobilization of material for regeneration. The action of BAL is a much more unspecific one. Through complex formation with heavy metal ions it interferes generally with cellular oxidations and acts accordingly as an unspecific toxic substance affecting the different cell types more uniformly than TEM.

9. *The time-graded field in relation to TEM treatment*

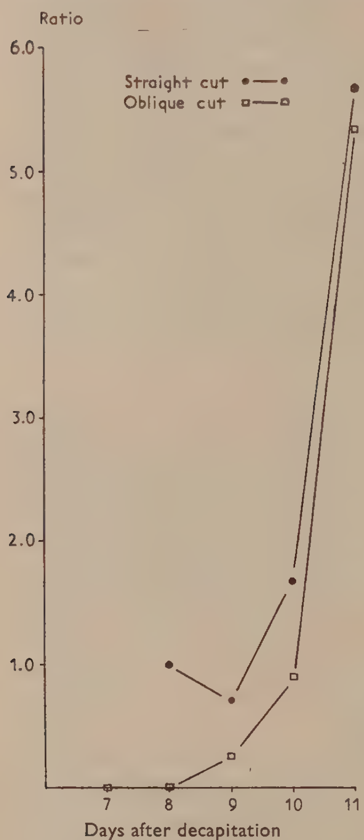
Brøndsted (1955) demonstrated that a cut in a planarian worm always exposes a point in the time-graded field from which regeneration will start earliest and most vigorously. This point will later be the starting-point of inhibiting forces, suppressing in other parts of the blastema the formation of structure similar to that in the high point. This notion has been extended to explain bilaterality as a state of latent inhibition: 'Two symmetrical halves of a bilateral organism react



TEXT-FIG. 4. Regeneration rate of untreated animals decapitated with a straight and an oblique cut.

in fact like two animals working harmoniously together. Only if they check each other's ability to produce their own mirror image they are able to do so. A latent inhibition is thus continuously at work' (Brøndsted, 1956).

During the analysis of the action of TEM it was realized that the one-eye phenomenon described in the last section might be correlated with an interaction of TEM with the unknown dynamics involved in the time-graded field, especially the inhibiting forces. TEM might act as an inhibition mimic, reinforcing the natural inhibition emanating from the side regenerating one eye at the fastest rate.

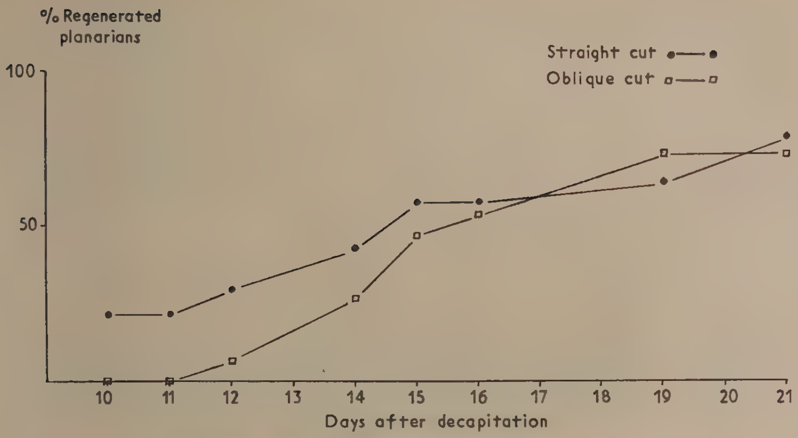


TEXT-FIG. 5. Ratio of number of animals with two regenerated eyes to number of animals with one regenerated eye after straight and oblique decapitation.

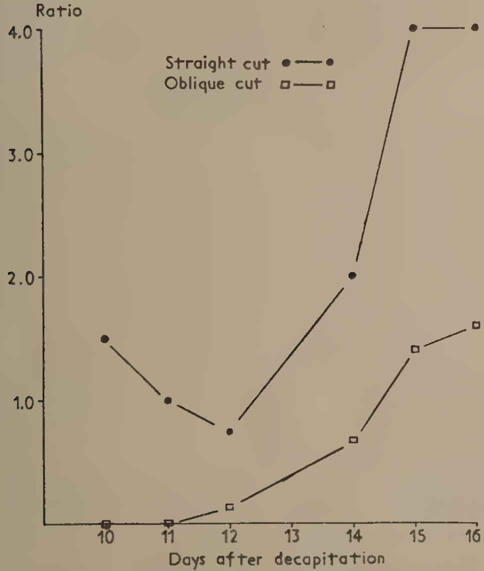
If this reinforcing of inhibition by TEM could be demonstrated, it might explain the rather variable time-lags between the occurrence of one lateral eye and the appearance of the contra-lateral one. A slight obliquity of the cut in the time-graded field will produce a difference in the ability to regenerate between the two bilateral halves of the animal, and consequently a difference in the time of appearance of the two eyes. Treatment with TEM should, according to hypothesis, enhance the difference by reinforcing the inhibition of one differentiating eye by the other. Another measure of the effect would be the ratio of animals with two regenerated eyes to animals with one, relative to the controls.

Two experiments were made to test this hypothesis. The first was a control experiment. Forty animals were decapitated, half of them with a transverse cut as usually applied, and the remaining with an oblique cut. The result is seen in Text-fig. 4. As might be expected, there is a decrease in the regeneration rate of the group with oblique decapitation, most pronounced in the first 2 or 3 days. Later there is a levelling out. Text-fig. 5 shows that in the first days of regeneration there is a preponderance of animals with one eye in the group exposed to oblique decapitation.

In the other experiment TEM was administered to two groups, each consisting of 15 animals. One group was decapitated with a transverse cut, the other with an oblique cut. TEM was administered just after decapitation, 15 mg./l. for 45 minutes. Text-fig. 6 shows that, as in the controls, there is a retardation in the regeneration rate, again pronounced in the first days of regeneration. Since regeneration is much slowed down, the number of days when a relative retardation of the obliquely decapitated relative to the transversely decapitated planarians



TEXT-FIG. 6. Regeneration rate of animals decapitated with a straight and an oblique cut. Treated with 15 mg. TEM per litre for 45 minutes just after decapitation.



TEXT-FIG. 7. Ratio of number of animals with two regenerated eyes to number of animals with one regenerated eye after decapitation with a straight and an oblique cut. Treated with 15 mg. TEM per litre for 45 minutes just after decapitation.

is observed is, of course, increased. Text-fig. 7 shows that the ratio between the number of animals with two regenerated eyes and those with one is similar to that of the controls, but, as with the regeneration rate, there is also here a somewhat prolonged period, relative to the controls, before levelling out starts.

There is thus no basis for the hypothesis that TEM has an inhibition-mimicking action reinforcing the inhibition emanating from the high point of regeneration. Moreover, the rather high scatter in the time-lag between appearance of one eye and the corresponding lateral one during TEM treatment is not the result of a more or less oblique cut in the time-graded field.

10. *Fission and TEM*

Fission in *P. vitta*, as a rule, takes place caudally to the pharynx. In a few cases, however, it has been observed to be pre-pharyngeal. Generally it takes place in an animal where the body caudal to the pharynx is long. Just behind the pharynx a constriction forms, and an accumulation of neoblasts is observed at each side of the constriction. Mitoses are seen too. After some time the constriction breaks, a blastema forms at each broken end, and regeneration occurs, most extensively, of course, in the posterior part. Fission is very often released after decapitation, after a latent period of a few days.

Fission is not much affected by TEM treatment. Only in the highest concentration used, 50 mg./l. continuously, is it quite abolished. Some figures may be given. All groups consisted of 20 animals. Fission occurred in 14 cases in controls, in 14 cases after administration of 0·5 mg./l., in 9 cases after 5 mg./l., in no cases after 50 mg./l. In another experiment fission was observed in 32 cases in controls, in 34 after administration of 0·01 mg./l., in 29 after administration of 0·1 mg./l., and in 26 after administration of 1 mg./l. With the exception of 0·1 and 0·01 mg./l., all concentrations were incompatible with regeneration, but fission was not significantly decreased except in the highest concentration used.

The development of the posterior fragments was always correlated with the regenerative response of the head-pieces. In one experiment involving treatment with TEM at 5 mg./l. continuously, the same number of fissions (27) was found in both the control and the experimental group. Eighteen days after decapitation most of the posterior fragments in the control group had completed their development to normal planarians, while no signs of regeneration were observed in the group treated with TEM. The posterior fragments had finally succumbed together with the head-pieces on the 22nd day after decapitation.

In an experiment involving short-term treatment, 50 mg./l. for 2 hours followed by decapitation 24 hours later, the rate of fission was the same as in the control group. The development of the fragments lagged somewhat behind the regeneration of the head-pieces, but as was the case with these, the posterior fragments, too, regenerated by morphallaxis, first a primary one with laying down of brain and eyes, and later a more extensive one involving remodelling of the whole animal.

The result of these experiments is that fission as such is not influenced (except after a most drastic treatment) by TEM. The regeneration of the posterior fragments resulting from fission follows the same pattern as that of head-pieces, and is thus greatly modified by exposure to TEM.

11. *Histological changes during TEM treatment*

As with the regeneration rate and the different morphogenetic activities, the histological changes of animals treated with TEM were rather variable. The same variability in histological pictures was observed after nitrogen mustard treatment of limb-buds of *Xenopus* larvae (Tschumi, 1954).

The neoblasts are the cells most sensitive to TEM. They may break down in two ways. They may become pycnotic and break down in the interphase or go through mitosis and break down in any of its phases, the chromosomes suffering fragmentation. They may also enlarge, forming giant cells with correspondingly enlarged nucleoli (Plate 1, fig. C). Later these giant cells break down too. A similar formation of giant cells after TEM treatment was observed by Töndury (1955), and after nitrogen mustard treatment by Tschumi (1954) and Bodenstein (1954). They are rather infrequently encountered in planarians. They have especially been observed 3 days after decapitation with continuous exposure to TEM. Neoblasts break down in the first 5–6 days after continuous treatment with TEM at 5 mg./l. Not all neoblasts are, however, equally sensitive. Normal mitoses may be found as previously described. A curious phenomenon was that the neoblasts forming the hypoplastic blastemas are relatively normal (Plate 2, fig. F), and they are the last neoblasts to break down. As a result of their disappearance regression of the blastema follows.

The neoblasts which have differentiated, e.g. to the hypoplastic brain ganglia, do not break down together with the neoblasts which have not differentiated, but degenerate with the nervous system later on.

Basophilia is decreased as a result of the depletion of the strongly basophilic neoblasts. Furthermore, the parenchyma appears looser and often vacuolated. Many granules appear in the parenchyma, most of them pyroninophilic. They may represent the depolymerized DNA from the degenerated nuclei. Some of them are, however, eosinophilic.

The different cell types show a great variation in sensitivity to TEM treatment. A sequence in break-down of the different tissues can be followed (Plate 2, figs. G and H): first the neoblasts, then the digestive tract (Plate 2, fig. G), then the nervous system, and finally the pharynx and epidermis. The epidermis frequently suffers metaplasia during the treatment, changing from cuboidal or columnar to squamous (Plate 1, fig. C).

These observations all refer to decapitated planarians treated with 5 mg./l. TEM continuously. Intact planarians treated with TEM were also examined. The same lesions were found, and the same differential sensitivity. Decapitated animals treated with lower, though definitely regeneration-retarding, doses of

TEM showed no abnormalities apart from hypoplastic blastemata, decreased mitotic activity, and pycnoses. No regression of the blastemata was found.

The PAS-reaction of Hotchkiss (Pearse, 1953) was used for examination of intact planarians exposed to TEM at 50 mg./l. continuously. The sections were counterstained with iron trioxyhematein (Plate 1, fig. D). The parenchyma is strongly PAS-positive, most intensely so the compact head parenchyma, the PAS-positive substance occurring partly in granules, partly homogeneously dispersed. The intestine and muscle fibres are also PAS-positive, somewhat less intensely than the parenchyma. The nervous system and brain are faintly positive, the substance occurring mainly homogeneously. Minot's glands in the intestine are nearly negative, the globules quite negative. The rhabdites are completely negative, although they are highly mucous.

Following treatment with TEM for 3 days there is no change, but after 6 days, while the parenchyma is still strongly positive, the intestine is almost negative. At this time there is still a preponderance of normal nuclei in the digestive system. After 10 days the PAS reaction is generally considerably decreased. The substance in the parenchyma is broken up into finer granules, and the intestine is almost completely negative. Most of the nuclei in the gut have disappeared at this time.

It is not clear from these observations whether the decrease in PAS-positive substance after intensive TEM treatment is an unspecific response preceding the total break-down of the tissues of the animal, or whether it is due to a specific interference with the metabolism of polysaccharides.

12. *Respiration after TEM treatment*

The respiration of intact planarians was studied after treatment with TEM for 2–4 days continuously in a concentration of 5 mg./l., a treatment incompatible

TABLE 2

Respiratory intensity of TEM-treated planarians (5 mg. TEM per litre continuously)

Controls		*TEM-treated*	
μg N	QO_2	μg N	QO_2
16·3	6·4	27·3	7·0
11·4	7·5	14·0	6·4
18·8	5·7	12·5	8·2
17·2	6·6	17·4	6·2
14·5	7·2	25·1	6·1
..	..	9·1	6·6
..	..	13·1	8·1
mean: 6·7±0·7		mean: 6·9±0·9	

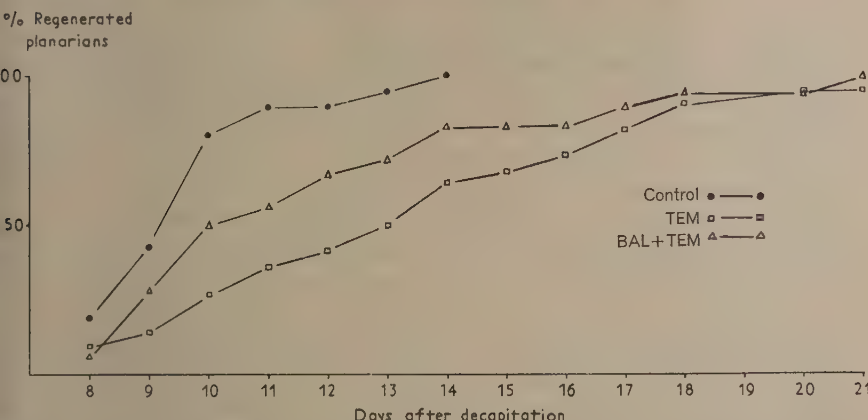
with regeneration. Respiration was determined in the Cartesian diver respirometer, one planarian being placed in each diver. The bottom-drop consisted of

TEM solution. For further technical details, see Pedersen (1956). As a reference for the oxygen consumption values, the total nitrogen of the animals was employed. The results are found in Table 2. It is evident that no change in respiratory intensity follows TEM treatment. A few values were obtained using a concentration of 50 mg./l. for 4 and 5 days. Still no change was observed.

These results agree with those of Holzer *et al*. (1956), who studied the influence of TEM on Ehrlich mouse ascites carcinoma. TEM had no action on respiration, but decreased glycolysis. The target of TEM according to these authors is the sulphydryl groups of triosephosphate dehydrogenase. Podolsky & Hutchens (1954) found no influence of the closely related nitrogen mustards on the respiration of *Chilomonas paramecium* and *Saccharomyces cerevisiae*.

13. *Influence of treatment with BAL*

Several experiments were made in order to influence the effect of TEM with a SH group containing substance, 2:3-dimercaptopropanol (BAL). This compound has been extensively used as a radiation-protective substance. It has been a much-favoured hypothesis that the site of action of radiation and of substances exhibiting radiomimetic actions is the SH groups.



TEXT-FIG. 8. Protective action of BAL administered for 45 minutes just after decapitation in a concentration of 5 mg./l. After rinsing, treatment with TEM at 5 mg./l. for a further 45 minutes.

BAL in itself is, as previously mentioned, very toxic and inhibits regeneration. When applied for a short time, however, it has no effect on regeneration. Its inhibitory action is also quickly abolished when the animals are transferred to pure tap-water. It does not exhibit the severe remote actions which TEM displays.

Several combinations of TEM and BAL were tried in the regeneration experiments. The animals were, of course, never treated simultaneously with the two compounds. Most of the experiments failed to show any influence of BAL on the regeneration-retarding effect of TEM. In one combination, however, a

definite effect was noted. Just after decapitation, BAL at 5 mg./l. was given to the animals for 45 minutes. After careful rinsing the animals were exposed to TEM at 5 mg./l. for 45 minutes. This procedure certainly involved some protection (Text-fig. 8). When TEM was administered before BAL was given, no protection was observed.

14. *The free amino acids during treatment with TEM*

In these experiments another planarian species, *Euplanaria polychroa*, was used. This is considerably larger than *P. vitta*. Only one or two of the large planarians are needed for a chromatogram. All the animals had been without food for a week in order to empty the digestive tract. For analysis they were quickly homogenized in ice-cold water (redistilled), centrifuged for 3 minutes, and deproteinized by boiling for 3 minutes in a water-bath with reflux. After centrifugation and transfer to clean centrifuge tubes they were kept in the ice-box (– 10° C.) until use. The extracts were run with two-dimensional paper chromatography as previously described.

In one experiment intact and decapitated planarians were treated continuously with TEM in a concentration of 50 mg./l. for 5 and 4 days respectively. Both intact and decapitated animals were used for control-extracts. Qualitatively there was no change except for a disappearance of tyrosine in the chromatograms from TEM-treated animals. The reaction of tyrosine is, however, very faint because of its low solubility in water, so its loss after treatment with TEM is hardly significant. A pronounced difference was, however, a very strong glutamine reaction in the extracts of treated animals, as compared with the controls, found in both the intact and decapitated planarians.

In other experiments intact planarians were treated with TEM at 5 mg./l. for 5 days or with 50 mg./l. for 2 days. No change in the distribution or amount of amino acids could be discerned in either case.

The significance of the elevated glutamine after intense treatment with TEM is not evident. It may possibly point to an interference with protein synthesis. Glutamine may be regarded as a reservoir for amino-groups, donating these to alpha-keto acids through a transamination reaction. In this way new amino acids for protein synthesis may be formed. An accumulation of glutamine might accordingly indicate an inhibition of protein synthesis.

DISCUSSION

The analysis of the morphogenetic partial processes of regeneration during treatment with TEM indicates that only one of the processes is affected, the recruitment of material for blastema formation, through inhibition of mitosis of neoblasts, and, in stronger concentrations, through a destruction of these cells. The influence on mitosis was determined partly directly, by counting, and partly by the fact that TEM acts at a specific time of regeneration related to the rhythm of mitotic activity. TEM has only slight effect on regeneration when adminis-

tered after the wave of mitosis has subsided; and it is more effective the earlier the application because more neoblasts and mitoses are affected. When the treatment is of low intensity (15 mg./l. for 45 minutes) an exposure on the day of decapitation strongly retards regeneration (Text-fig. 1) because mitosis is affected from the outset of regeneration. But if mitosis has been allowed to go on for 2 days (e.g. before the maximum of the mitotic wave has set in) regeneration is unaffected; the preliminary proliferation of neoblasts has been sufficient to deliver material for regeneration. After a more drastic treatment (e.g. 5 mg./l. TEM for 3 days, Text-fig. 3), the same kind of response is encountered, but far more neoblasts and mitoses are affected, and accordingly the time relations have changed. Treatment after the subsidence of the mitotic maximum only results in a slight retardation of regeneration.

Except for treatment with high and lethal doses of TEM the other morphogenetic processes constituting regeneration are unaffected. Migration, differentiation, induction, inhibition (fission too), go on, but are often modified as a consequence of the inhibition of mitosis. Far more modified, however, is the balance between the two modes of regeneration, epimorphosis and morphallaxis.

A similar dissociation between morphogenetic processes involving the inhibition of mitosis, but no direct affect on differentiation, migration, and induction, has been described after treatment with nitrogen mustard, a substance very similar to TEM in its actions. Nieuwkoop & Lehmann (1952) found such a dissociation in *Triton* embryos, Tschumi (1954) in hind-limb-buds of *Xenopus* larvae.

The suppression of blastema formation resulting from TEM treatment manifests itself in two interesting morphogenetic modifications, the first bearing on the balance between epimorphosis and morphallaxis, the other on competition and inhibition during regeneration.

Treatment with TEM involves inhibition of epimorphosis since the formation of a blastema is suppressed. Nevertheless, regeneration occurs. Epimorphosis is dispensed with, and regeneration takes place through a somewhat modified morphallaxis. The process may roughly be divided into two phases. Firstly, a preliminary morphallaxis sets in with the differentiation of a hypoplastic brain and one or two eyes. Secondly, an extensive morphallaxis gradually and slowly ensues, resulting in a completely normal animal. This is, of course, conditional on an interrupted and non-lethal TEM administration. The preliminary morphallaxis with partial and hypoplastic regeneration is possible, however, even after continuous treatment with TEM in lethal concentration (5 mg./l.). Regeneration of a hypoplastic brain and one lateral eye are often observed under these circumstances, but regeneration is never complete, and regression of the regenerated structures occurs together with the general regression ending in the death of the animal.

An interesting fact is that this preliminary morphallaxis goes on without

concomitant mitosis. The origin of material for the process is not certain, but microscopical studies suggest that the cells drawn upon are partly the neoblasts surviving the TEM treatment. Possibly many neoblasts are rather early determined, and accordingly lose their extreme sensitivity to TEM and contribute to the cellular material for regeneration together with the surviving non-determined neoblasts.

The later extensive secondary morphallaxis occurring after non-lethal treatment involves the activity of neoblasts. According to the severity of treatment the process goes on slowly or quickly. This fact possibly reflects a more or less slow regeneration and proliferation of neoblasts. Mitosis certainly plays an important role in this secondary morphallaxis.

It can be concluded that in *P. vitta* a dissociation between epimorphic and morphallactic modes of regeneration has been achieved. The cellular basis of both processes, however, is the same: totipotent parenchymatous cells, the neoblasts. Mitosis of neoblasts plays an important part in both processes, most evidently in epimorphosis. A preliminary morphallaxis resulting in hypoplastic structures may take place without concomitant mitosis, but the later extensive morphallaxis resulting in a normal animal most probably involves replacement of the depleted neoblasts through proliferation.

The other interesting morphogenetic consequence of suppression of mitosis and depletion of neoblasts during regeneration bears on inhibitory forces and competition. The fundamental significance of these two processes in the control of growth and differentiation in morphogenetic systems has been established through the works of Spiegelman (1945), Rose (1952, 1955), Weiss (1955), Lehmann (1953), Tardent (1956), Steinberg (1954), and others. In planarian regeneration they have been considered by Brøndsted (1956) and Lender (1955).

The one-eye phenomenon described in section 8 may be explained in terms of competition and inhibition. Brøndsted (1956) demonstrated inhibitory forces between the two lateral halves of planarians and accordingly between the symmetrical organs, e.g. the brain ganglia and eyes. Lender (1955) proved the existence of substances present in extracts of heads of the planarian *Polycelis nigra* which inhibit brain regeneration. The inhibition exercised by the extracts is considered to be specific. In the presence of extracts from the trunk, brain regeneration is only slightly inhibited.

As a consequence of treatment with TEM, regeneration material is scarce. One side of the planarian starts its regeneration faster than the contra-lateral side and perhaps seizes the majority of neoblasts present. Accordingly, a competition for neoblasts perhaps occurs, resulting in the dominance of the region with greater morphogenetic activity over the contra-lateral region. The dominance explains the rather frequently observed phenomenon that the dominating lateral half is provided with a larger brain ganglion, and the eye appearing in this region grows in size without formation of an eye-spot in the contra-lateral half. It should be stressed that this inhibition is not of differentiation as such: it

is an inhibition of accumulation of neoblasts. As soon as sufficient neoblasts have accumulated in the dominated half, differentiation begins.

As to the mechanisms through which dominance exercises its action, an action manifesting itself as inhibition, one aspect of it is certainly an inhibition through chemical substances. For *Hydra* and *Tubularia* this was proved by Tardent (1955, 1956). For planarians it was made probable by Lender (1955). It has not been possible to prove an interference with these substances, TEM acting as an inhibition mimic. As this aspect of dominance is the only one accessible with present knowledge, the function of TEM in its basic influence on competition is still unknown.

The few biochemical observations made in this study point to an interference of TEM with DNA metabolism and protein metabolism, while no action on respiration was found. If this is so, the chemical systems interfered with are so fundamental that an alteration of them might be expected to have serious morphogenetic consequences. On the other hand, this interference suggests that TEM should lack specificity. Probably many other anti-mitotic and cytotoxic substances with similar points of attack might give similar morphogenetic modifications.

Tschumi (1953, 1954) made some interesting studies on the consequences of experimental reduction of blastemas of limb-buds in *Xenopus* by treatment with nitrogen mustard. Legs with syndactylism, rudimentary, or missing toes developed as a result of competition between the primordia of the toes. The results obtained in the present paper fall in line with those of Tschumi. In both cases inhibition phenomena are encountered following competition for cellular material, this material being scarce after treatment with a cytotoxic and anti-mitotic substance.

SUMMARY

1. The action of an anti-neoplastic substance, triethylene melamine (TEM) on the morphogenetic processes of head regeneration in the planarian *Planaria vitta* has been studied.
2. A description of the normal regeneration is first given.
3. TEM in very low concentrations strongly retards or blocks regeneration of two eyes.
4. Administered continuously, a high toxicity is observed. Exposure to high concentrations for a short time involves severe retardation of regeneration without any mortality.
5. A phase-specificity is observed. TEM in low concentrations only acts in the first phase of regeneration, in higher concentrations in the middle phase too, but no action is encountered in the late phase of regeneration.
6. The phase specificity is not due to a failure of penetration of the substance.
7. TEM strongly affects mitosis of neoblasts, and this is its primary action on the regenerative process.

8. No action on cellular migration was observed.

9. A dissociation between epimorphosis and morphallactic regeneration is possible following treatment with TEM.

10. Differentiation and induction are not directly influenced by TEM, but may be modified as a result of depletion of neoblasts.

11. A competition of the two symmetrical lateral halves for neoblasts is encountered, resulting in animals with only one lateral eye. The phenomenon is quite distinct from cyclopia.

12. Fission as such is unaffected by TEM. The regeneration of the posterior fragments follows the same pattern as the head-pieces.

13. Histological changes following TEM treatment are described.

14. The PAS reaction is depressed after intense treatment with TEM, the intestine being first depleted of PAS-positive material.

15. Respiratory intensity is unaffected by TEM.

16. A partial protection is obtained by pretreatment of the animals with BAL (2:3-dimercaptopropanol).

17. The free amino acids were studied after TEM treatment. Only one change is observed, a highly elevated glutamine reaction.

18. The significance of epimorphosis and morphallaxis, and the respective role played by mitosis and neoblasts in these two modes of regeneration are discussed. Competition and inhibition phenomena during regeneration are briefly discussed.

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Sagittal section. Helly-fixed animal stained with azan. Morphallaxis, 25 days after decapitation. Treated with TEM. No blastema. Brain (*A*) morphallactically formed with distinct nerve fibres. Cranial to the brain further regional brain differentiation is going on (*B*). Magnification ×470.

FIG. B. Sagittal section. Fixed in acetic-alcohol. Giemsa stain followed by acetone-dehydration. TEM treated. Hypoplastic brain ganglion (*A*). Few neoblasts. Magnification ×470.

FIG. C. Sagittal section. Fixed in Carnoy 3 days after decapitation. Treated with TEM. Methyl green pyronin. Giant cells. One cell focused on the enlarged nucleolus (*A*). Metaplastic squamous epithelium (*B*). Magnification ×1,170.

FIG. D. Sagittal section. Normal planarian fixed in buffered formalin. PAS reaction plus iron trioxyhematein. *A*: intestine. *B*: ventral nerve cord. *C*: parenchyma. *D*: Minot's gland. Magnification ×375.

PLATE 2

FIG. E. Sagittal section. Carnoy-fixation. Methyl green pyronin. Normal blastema 5 days after decapitation. Magnification ×940.

FIG. F. Sagittal section. Carnoy fixation. Methyl green pyronin. Hypoplastic blastema 5 days after decapitation. Continuous treatment with TEM, 5 mg./l. Magnification ×940.

FIG. G. Sagittal section. Helly-fixation. Azan. Intact planarian treated for 6 days with TEM, 50 mg./l. Break-down of the digestive tract, degenerating nuclei. In the centre a Minot gland. Magnification ×940.

FIG. H. Same section as fig. G. Intact brain with normal nuclei. *A*: nerve fibres in the brain ganglion. *B*: cut muscle fibres. *C*: eye-spot. Magnification ×940.

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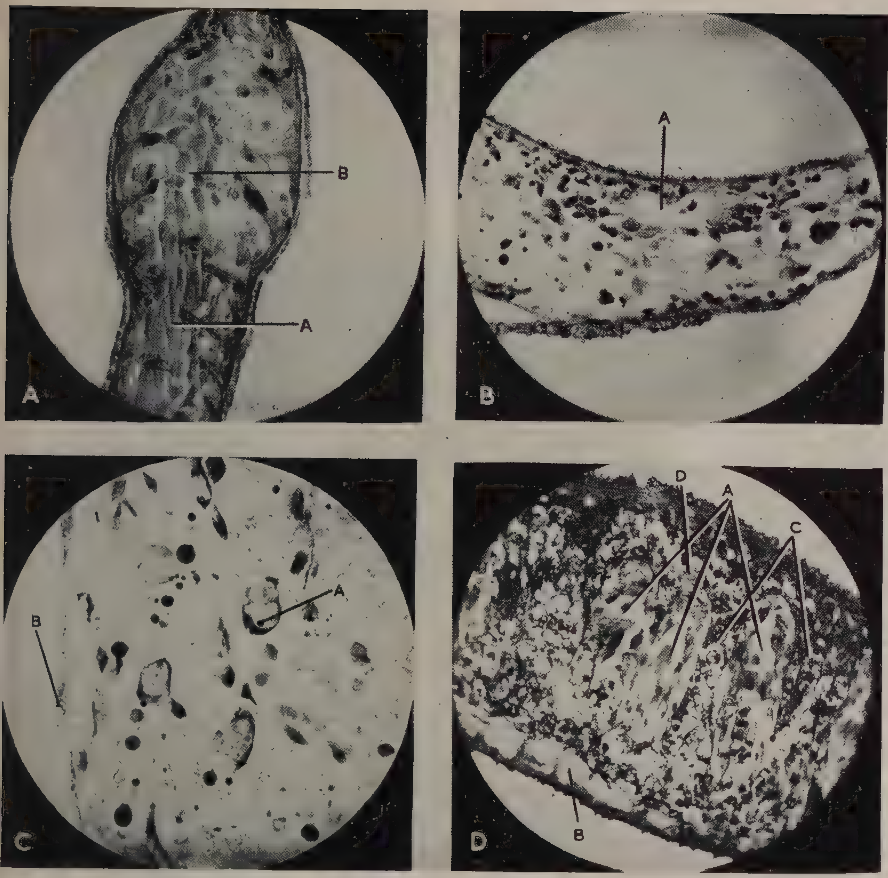
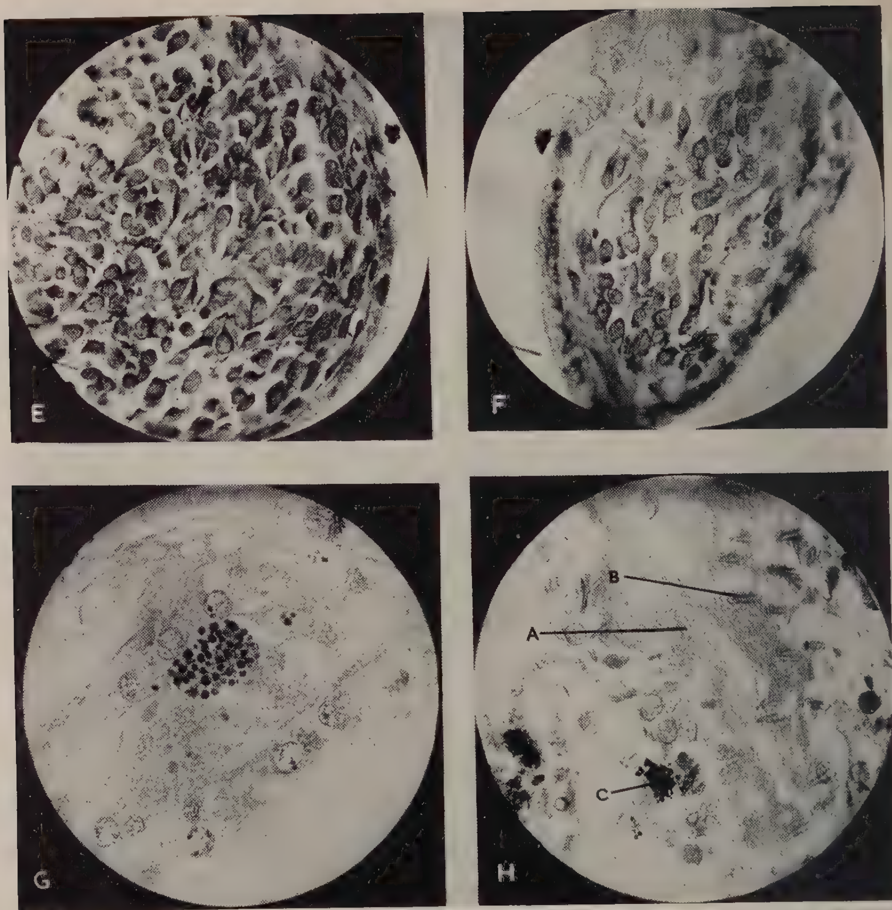


K. J. PEDERSEN

Plate 1



K. J. PEDERSEN

Plate 2

Recherches sur l'action tératogène du refroidissement temporaire de l'œuf de Poule au cours de l'incubation

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AVEC TROIS PLANCHES

De très nombreux auteurs ont signalé les effets tératogènes de divers agents physiques sur l'œuf de Poule (incubation à des températures élevées ou basses, vernissage des œufs, secouage, prélèvement d'albumine, réalisation d'une fenêtre dans la coquille, radiations diverses, ultra sons etc.) avant ou pendant les premiers jours de l'incubation. Nous avons cherché à savoir si des malformations différentes de celles qui avaient été ainsi obtenues pouvaient l'être après la soixante-douzième heure de l'incubation. Nous avons choisi comme agent un choc thermique qui n'a pas d'action tératogène dans les premiers jours de l'incubation.

TECHNIQUE

Elle a essentiellement consisté à retirer de la couveuse des œufs de Poules Leghorn blanches à diverses périodes de l'incubation et à les laisser séjourner pendant un certain temps dans un local dont la température a varié de 17 à 24 degrés. Cette technique paraît d'après Dareste (1891) avoir été imaginée par Panum (1860) qui retirait les œufs de la couveuse après 2 à 3 jours d'incubation et les laissait exposés pendant quelques heures à la température de l'air ambiant. Elle a été reprise par Dareste qui refroidissait des œufs ayant atteint la fin du quatrième jour de l'incubation. Cet auteur a conclu à l'absence du pouvoir tératogène de cette technique. Ce sont vraisemblablement ces conclusions négatives qui ont empêché les auteurs qui l'ont suivi de faire les recherches qui, en variant les conditions expérimentales, leur en auraient montré ce pouvoir tératogène.

Les conditions dans lesquelles nous nous sommes placés sont exactement les suivantes: Une fenêtre ronde d'environ 15 mm. de diamètre a été ouverte dans la coquille après 72 heures d'incubation et fermée par une lamelle de ruban adhésif. Les malformations visibles à la loupe des embryons morts ou vivants à ce moment ont été notées et tous les œufs renfermant des embryons vivants ont

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été remis temporairement à la couveuse puis au froid à des périodes variées de l'incubation.

Le nombre total des œufs mis en expérience a été de 699. Neuf variétés d'expériences ont été faites, différant seulement par le jour de l'incubation où l'œuf a été mis au froid et le temps où il y a séjourné. L'examen des embryons a été fait immédiatement avant la mise au froid et les morts éliminés de l'expérience. Un autre examen a été fait immédiatement avant la remise à la couveuse après action du froid. Il a montré que la circulation était arrêtée mais que le cœur pouvait encore battre chez certains embryons.

RÉSULTATS

Effet létal

Un examen fait 24 heures après la remise en couveuse a montré que la circulation sanguine arrêtée pendant le séjour au froid ne s'était pas rétablie chez un assez grand nombre d'embryons. Les tableaux 1 et 2 donnent les périodes du développement pendant lesquelles les œufs ont séjourné au froid au cours de

TABLEAU 1

Tableaux résumant les effets létaux et tératogènes du séjour au froid des œufs pendant 2 à 3 jours entre le 3e et le 8e jour de l'incubation

Période de séjour au froid, jours	*Nombre d'embryons mis au froid*	*Morts 24 h. après remise en couveuse*	*Vivants au 10e jour*	*Ablépharie*	*Malformation des membres*	*Bec de lièvre*	*Atrophie du bec*	*Buphtalmos*
3e au 5e	55	38,7	52,7	0	0	0	0	0
4e au 6e	87	17,2	63,2	10,3	5,7	5,7	0	5,7
5e au 7e	99	38,3	43,4	13,1	13	3	0	6
6e au 8e	81	25,9	58	3,7	4,9	0	14,8	11,1
7e au 9e	86	28	58,1	0	17,3	0	12	6,6
8e au 10e	85	39,4	60,5	0	20	0	0	0

TABLEAU 2

3e au 6e	42	54,7	14	2,3	0	0	0	0
4e au 7e	89	65,1	21,3	11,2	8,9	11,1	0	4,4
5e au 8e	75	77,6	21,3	11,9	29	2,6	6,6	5,3

Pour chaque malformation les chiffres indiquent le pourcentage des embryons qui en étaient porteurs. Pour l'effet létal les chiffres indiquent le pourcentage des embryons trouvés morts 24 heures après la remise en couveuse consécutivement au séjour au froid.

nos 9 variétés d'expériences (2 jours dans le tableau 1, 3 jours dans le tableau 2) et le nombre d'œufs mis en expériences renfermant des embryons vivants. Ces tableaux donnent aussi le pourcentage des embryons morts 24 heures après la remise en couveuse. Cette proportion peut servir à évaluer le pouvoir létal de la technique. Il apparaît approximativement le même après deux jours de refroidissement quel que soit le jour où a lieu la mise au froid (vers 30 pour cent) mais plus faible du 4e au 6e jour. Le refroidissement pendant 3 jours a doublé

approximativement la mortalité (tableau 2) et la fréquence augmente lorsqu'avance le stade auquel l'œuf est refroidi.

Les tableaux donnent aussi le pourcentage d'œufs vivants au dixième jour de l'incubation. Dans toutes les variétés d'expériences certains embryons sont morts entre le vingtième et le vingt-cinquième jour de l'incubation, d'autres sont éclos avec ou sans malformations. Des statistiques n'ont pas été faites à ce sujet parce que de nombreux embryons ont été prélevés vivants entre le dixième et le vingtième jour.

Effet tératogène

Parmi les malformations dont étaient porteurs les embryons obtenus dans nos expériences après action du froid, certaines faisaient partie de celles qu'on rencontre après mise en œuvre d'agents physiques avant la soixante-douzième heure de l'incubation. Elles étaient peu nombreuses. Quatre acléiencéphales (habituellement dénommés anencéphales), 2 omphalocéphales, 1 spina bifida, 1 ectrosome, 1 anoure, 1 symèle, 1 monstre double. Ces malformations peuvent être considérées comme des malformations spontanées étant donné leur faible pourcentage.

Les autres malformations obtenues sont très rares à l'état spontané, nous les grouperons sous quatre chefs: ablépharie, malformations des membres, malformations du bec, œdèmes et hémorragies localisés.

A. *Ablépharie* (Pl. 1 et 2, fig. 1 à 7)

L'absence totale des deux paupières a été observée ainsi que des cas d'ablépharie localisée caractérisés par l'absence d'une plus ou moins grande partie des paupières allant d'une petite incisure au remplacement d'une paupière par un petit bouton. L'ablépharie peut être localisée à la région moyenne de l'une des deux paupières ou consister dans l'absence de la commissure antérieure ou de la postérieure. L'ablépharie peut aussi être localisée à un seul œil ou être bilatérale et dans ce cas elle peut avoir exactement la même localisation sur les deux yeux. Nous avons divisé tous les cas observés en 4 catégories: absence totale comprenant les cas où ne persiste qu'un petit bouton palpébral; absence de la commissure antérieure; absence de la commissure postérieure; absence de la partie moyenne comprenant tous les cas où cette partie moyenne fait défaut quelle que soit son étendue. Les résultats sont consignés dans le tableau 3 qui donne le pourcentage de ces quatre catégories pour chaque variété d'expérience. Les variétés d'expériences dans lesquelles des cas d'ablépharie ont été constatés sont au nombre de 6 comprenant 476 embryons. L'absence totale des paupières a été constatée dans 17 cas, celle de la commissure antérieure dans 11 cas, celle de la commissure postérieure dans 49 cas, et les lésions de la région moyenne dans 16 cas. La malformation la plus fréquente est l'absence de la commissure postérieure, son maximum de fréquence est apparu dans les expériences de refroidissement de l'œuf du cinquième au septième jour de l'incubation.

Dans leur ensemble ces expériences nous ont donné 41 embryons porteurs

d'ablépharie. Le nombre d'yeux ablépharíques était de 68, chez 27 embryons l'ablépharie était donc bilatérale. Dans 14 cas où l'ablépharie était unilatérale 7 cas affectaient l'œil gauche et 7 le droit.

TABLEAU 3

La statistique suivante a été établie en tenant compte du fait que dans chaque œil la région commissurale postérieure de même que l'antérieure est formée de deux parties l'une supérieure et l'autre inférieure. Ces deux parties peuvent faire défaut ou seulement l'une d'entre elles. Quand les deux parties d'une commissure sont absentes on a compté 2 cas d'ablépharie localisée. Quant à l'absence des paupières le nombre de cas signalés est celui des paupières absentes sur les deux yeux

Période de séjour au froid, jours	*Nombre d'embryons mis au froid*	*Absence totale*			*Absence commissure antérieure*			*Absence commissure postérieure*			*Absence partie moyenne*	
		2 paup.	*Sup.*	*Inf.*	*2 paup.*	*Sup.*	*Inf.*	*2 paup.*	*Sup.*	*Inf.*	*Sup.*	*Inf.*
3ᵉ au 5ᵉ	55	0	0	0	0	0	0	0	0	0	0	0
3ᵉ au 6ᵉ	42	0	0	0	0	0	0	0	0	0	4,7	0
4ᵉ au 6ᵉ	87	5,7	2,2	0	1,2	0	0	2,2	4,5	0	3,4	0
4ᵉ au 7ᵉ	89	4,4	0	1,1	2,2	0	1,1	4,4	5,6	0	3,3	0
5ᵉ au 7ᵉ	99	0	0	3	1	2	1	15,1	6	2	1	1
5ᵉ au 8ᵉ	75	0	0	2,6	1,3	0	2,6	5,3	5,3	0	4	0
6ᵉ au 8ᵉ	81	0	0	0	0	0	0	2,4	0	1,2	0	0
7ᵉ au 9ᵉ	86	0	0	0	0	0	0	0	0	0	0	0
8ᵉ au 10ᵉ	85	0	0	0	0	0	0	0	0	0	0	0

Une autre statistique porte sur tous les cas d'ablépharie relevés sur les deux paupières de tous les yeux (par exemple l'absence de la commissure antérieure et celle de la postérieure sur un œil est comptée comme 4 cas d'ablépharie). Dans ces conditions nous avons relevé 153 cas d'ablépharie, 83 pour la paupière supérieure et 70 pour l'inférieure, 49 à droite et 34 à gauche pour la paupière supérieure, 37 à droite et 33 à gauche pour l'inférieure.

L'ablépharie a été observée dans toutes les variétés d'expériences sauf dans celles où les œufs ont été refroidis du troisième au cinquième jour et celles où ils l'ont été du septième au neuvième et du huitième au dixième. Le maximum des cas a été observé après refroidissement du cinquième au septième jour où ont été trouvés 13 embryons ablépharíques, 21 yeux avec ablépharie et un total de 78 cas d'ablépharie, c'est-à-dire plus de la moitié de tous les cas observés dans nos expériences puisqu'ils étaient de 153.

Le pourcentage des embryons ablépharíques a au contraire été à peu près le même dans les quatre variétés d'expériences où il a été le plus élevé (4ᵉ jour au 6ᵉ jour, 4ᵉ au 7ᵉ, 5ᵉ au 7ᵉ, 5ᵉ au 8ᵉ). Voir tableau 3.

B. *Malformation des membres* (Pl. 2 et 3, fig. 10 à 17)

Les malformations suivantes ont été observées: Absence totale de l'aile ou de

la patte ou son remplacement par un petit bourgeon (ectomélie); absence de la partie distale du membre (hémimélie); atrophie d'un ou de plusieurs orteils soudés ou non entre eux; absence du deuxième doigt, absence du premier orteil (ectrodactylie), absence de la phalange unguéale du deuxième et du quatrième orteil; accollement entre le premier et le deuxième orteil ou entre le deuxième et le troisième (syndactylie par accollement); union entre le troisième et le quatrième orteil par une membrane ne faisant défaut qu'au niveau de la phalange unguéale (syndactylie membraneuse); déformation d'orteils caractérisée par la position de la phalange unguéale à angle droit sur la précédente; dédoublement du premier et du deuxième orteil dans sa partie distale, dédoublement du deuxième doigt (polydactylie) (Pl. 3, fig. 16). Des cas de malformations des membres ont été observés dans toutes les variétés d'expériences sauf dans celles où la mise au froid avait été faite le troisième jour de l'incubation (voir tableaux 1 et 2). Les déformations de la phalange unguéale (clinodactylie) n'ont été observées que dans les expériences où la mise au froid avait été faite au huitième jour.

Au total 72 cas de malformation des membres ont été observés, 20 portant sur le membre supérieur et 52 sur le membre inférieur. Pour le membre supérieur 5 à droite et 15 à gauche et pour l'inférieur 25 cas à droite et 27 à gauche. Certaines malformations existaient symétriquement des deux côtés: ce sont la syndactylie par accollement du premier avec le deuxième orteil, l'absence de la phalange unguéale du quatrième orteil et la syndactylie membraneuse entre le troisième et le quatrième orteil. Les cas de polydactylie étaient au nombre de 5: deux cas de dédoublement du premier orteil obtenus après refroidissement des œufs du quatrième au sixième jour et du quatrième au septième, un cas de dédoublement du deuxième orteil après refroidissement du huitième au dixième et deux cas de dédoublement du deuxième doigt par refroidissement du septième au neuvième.

C. *Malformations du bec*

Ces malformations portaient sur le demi-bec inférieur ou sur le demi-bec supérieur ou sur ces deux parties.

(*a*) *Malformations du demi-bec supérieur* (Pl. 1 et 2, fig. 3, 5, 7, 8). Nous avons observé le bec de lièvre, le colobome de la face et l'atrophie. Le bec de lièvre est caractérisé par l'absence de la partie formée par le bourgeon nasal interne. Treize cas en ont été observés: 2 bilatéraux, 7 à droite, et 4 à gauche. Cette malformation n'est apparue qu'après refroidissement du quatrième au sixième jour, du quatrième au septième, du cinquième au septième et du cinquième au huitième (Pl. 1, fig. 3 et 5). Un cas de colobome de la face dû à l'atrophie des bourgeons nasaux externe et interne a aussi été observé du côté gauche après refroidissement des œufs du cinquième au septième jour.

Dans les cas d'atrophie du demi-bec supérieur (Pl. 2, fig. 7 et 8) celle-ci portait sur la partie du bec située en avant des narines et formée par le prémaxillaire. 4 cas en ont été observés dont 3 accompagnant l'atrophie du demi-bec inférieur;

3 de ces 4 cas sont apparus après refroidissement des œufs du sixième au huitième jour et un après refroidissement du cinquième au huitième.

(*b*) *Malformations du demi-bec inférieur* (Pl. 1 et 2, fig. 1, 4, 5, 8, 9). Quinze cas de cette atrophie ont été observés dans lesquels la fente palatine était largement mise à nu. Dans certains de ces cas les deux ébauches osseuses ne s'étaient pas fusionnées sur la ligne médiane et étaient réunies l'une à l'autre par une partie membraneuse. Un cas a été observé après refroidissement des œufs du cinquième au septième jour; 5 cas après refroidissement du cinquième au huitième et 9 cas après refroidissement du sixième au huitième jour.

D. *Hémorragies et œdèmes localisés* (Pl. 3, fig. 17 à 20)

Des boules sanguines ou œdémateuses ont été observées à l'extrémité distale ou le long des membres, sur la tête et sur le corps. Celles qui siégeaient sur les membres s'accompagnaient parfois de leur malformation. Les autres avaient seulement pour effet d'empêcher la formation du duvet. Sur 72 cas de malformation des membres 16 étaient accompagnés d'hémorragies (22,2 pour cent) qui en étaient vraisemblablement la cause. Ces hémorragies tératogènes localisées sont apparues dans toutes les expériences sauf dans celles où le refroidissement des œufs a eu lieu du sixième au huitième jour. Celles qui se sont produits après refroidissement au quatrième et au cinquième jour ont été les plus nombreuses et se sont accompagnées des malformations les plus graves (ectromélie et hémimélie). Les hémorragies localisées à la tête et au tronc sont apparues seulement dans les expériences où les œufs avaient été refroidis aux sixième, septième ou huitième jour, elles ont dans tous les cas empêché la formation du duvet à leur niveau.

Les boules hémorragiques ne renfermaient pas toutes du sang pur mais mélangé en quantités variables avec du liquide d'œdème et on rencontrait des boules œdémateuses et des boules sanguines au contact les unes des autres (Pl. 3, fig. 20).

De grosses boules œdémateuses ont aussi été observées au devant de l'œil constituant une saillie (buphtalmos) qui écartait les paupières normalement développées ou coexistait avec de l'ablépharie. Dans certains cas ces boules œdémateuses oculaires renfermaient aussi du sang (Pl. 1, fig. 6).

La fréquence avec laquelle sont apparus ces cas de buphtalmos est donnée dans le tableau 1. Il montre leur apparition dans toutes les variétés d'expériences sauf celles où le refroidissement de l'œuf a eu lieu le troisième et le huitième jour de l'incubation.

DISCUSSION

Effet létal

Nos résultats résumés dans les deux premiers tableaux mettent en évidence une forte action létale de la technique utilisée (en moyenne 31 pour cent de mortalité des embryons 24 heures après leur remise à la couveuse à la sortie du local froid). Elle a cependant permis à une forte proportion d'embryons de

dépasser le dixième jour de l'incubation (en moyenne 56 pour cent) et à certains d'entre eux d'atteindre vivants la fin de la période d'incubation et même d'éclore, quoique porteurs de malformations.

La comparaison entre les tableaux 1 et 2 montre que l'effet létal a été beaucoup plus grand quand les œufs ont séjourné hors de la couveuse pendant 3 jours au lieu de deux. La proportion des morts le lendemain de la remise à la couveuse après séjour au froid pendant 2 jours a été en effet de 31,4 pour cent, tandis qu'elle s'est élevée à 65,8 pour cent quand ce séjour a eu une durée de 3 jours. La proportion des embryons vivants au dixième jour est tombée de 53,1 à 18,8 pour cent.

Effet tératogène

Au point de vue des malformations, le bec de lièvre est apparu comme ayant son maximum le plus précoce parmi les différentes variétés de malformations obtenues (refroidissement du quatrième au sixième jour), celui de l'ablépharie vient ensuite (refroidissement du cinquième au septième jour), puis celui de l'atrophie du bec (refroidissement du sixième au huitième jour). Quant aux malformations des membres, débutant avec le refroidissement du quatrième au sixième jour, leur proportion s'est progressivement élevée sauf après le refroidissement entre le sixième et le huitième jour où elle a au contraire été le plus faible.

Ces résultats mettent en évidence les stades les plus favorables pour obtenir une malformation déterminée après la soixante-douzième heure de l'incubation. A ce sujet nous avons aussi constaté que sur 13 cas de syndactylie avec membranes, 2 avaient été obtenus après refroidissement du cinquième au septième jour; 5 après refroidissement du septième au neuvième et 6 après refroidissement du huitième au dixième. Tous les cas de syndactylie par accollement de deux orteils voisins l'un de l'autre ont été obtenus après refroidissement du quatrième au sixième et au septième jour et du cinquième au septième jour. Ce dernier stade est le seul où les deux variétés de syndactylie ont été réalisées, il est aussi le plus avancé auquel s'est produite la syndactylie par accollement et le plus jeune où sont apparus les cas de syndactylie membraneuse.

Malformations obtenues chez des Mammifères

Les cas d'hémorragies et d'œdèmes localisés que nous avons obtenus nous paraissent devoir être rapprochés de ceux qu'ont signalés plusieurs auteurs chez des embryons de Mammifères par injections de substances chimiques ou des carences.

Jost (1953) a injecté à des foetus de rat de 16 à 17 jours directement à travers la paroi utérine des solutions de diverses substances hormonales qui provoquent au niveau des extrémités (membre, queue, langue, etc.) des hémorragies suivies de nécroses pouvant entraîner l'amputation congénitale ou la déformation des extrémités. Des injections faites à des embryons plus jeunes ont fait apparaître des ampoules claires sur le dos ou la tête. Certaines des conclusions de cet auteur

montrent que les faits qu'il a observés sont les mêmes que ceux que nous signalons chez l'embryon de Poule après action du refroidissement des œufs. (1) Les lésions apparaissent à la suite d'une série de processus comportant successivement de l'œdème avec développement parfois d'ampoules claires puis de volumineuses ampoules hémorragiques. (2) Des hémorragies peuvent apparaître d'une manière inconstante sur diverses parties du corps. (3) Nous n'avons pas observé comme Jost des hémorragies à l'extrémité de la queue, mais seulement aux membres. D'après les résultats de Jost, ce fait pourrait être dû à ce que le refroidissement temporaire de l'œuf soit un facteur tératogène de trop faible puissance, cet auteur signale en effet que pour léser la queue il faut injecter une dose de substance supérieure à celle qui est nécessaire pour frapper les membres et une encore plus forte pour léser la langue.

Lefebvres-Boisselot (1951) a observé chez des embryons de rat dont les mères étaient carencées en acide pantothénique des hémorragies au niveau de l'encéphale et des méninges et assez souvent à l'extrémité des membres, surtout des membres postérieurs, ces hémorragies durcissaient et gonflaient les tissus et déformaient les doigts, mais l'auteur n'a constaté aucune malformation.

Martinet (1953) a observé chez des embryons de rat des hémorragies situées habituellement au niveau des pattes postérieures, mais aussi des pattes antérieures et à la queue. Ces hémorragies ont été observées dans de fortes proportions (20 à 36 pour cent) après carence en diverses vitamines (vitamines K, E, P, et B), l'acide folique et l'acide pantothénique. Ces hémorragies gonflent et déforment les tissus, dans un cas elles ont réalisé une amputation. Diverses expériences ont amené Martinet à la conclusion que les hémorragies sont dues à une insuffisance en acide linoléique qui, injecté à faible dose, a empêché ces hémorragies d'apparaître.

Mécanisme tératogène

Au point de vue du mécanisme de réalisation des malformations des membres, obtenues à la suite des injections qu'il a faites à des foetus de rat, Jost (1953) pense qu'elles résultent de la lésion de parties déjà constituées, conduisant à l'amputation congénitale, lorsque la régression est assez étendue, ou à des malformations diverses telles que le pied bot, la syndactylie ou la polydactylie, dans les cas où seule une partie de l'ébauche est atteinte.

L'auteur appuie son opinion sur les travaux de Greene & Saxton (1939) et Inman (1941) qui considèrent l'absence héréditaire de doigts ou d'un ou plusieurs membres et parfois de l'oreille comme résultant d'extravasations sanguines et d'hémorragies qui conduisent à la nécrose puis à la chute avant la naissance de la partie de l'extrémité intéressée. Il s'appuie aussi sur le travail de Bagg (1929) qui a montré que chez la souris dans la lignée de Little et Bagg, les lésions des membres généralement associées à des lésions oculaires, le pied bot et la polydactylie, sont précédées par l'apparition d'ampoules claires qui deviennent ensuite hémorragiques; faits qu'a confirmés Plagens (1933).

En ce qui concerne les malformations que nous avons obtenues chez l'embryon de Poule, certaines des malformations des membres sont dues à des hémorragies localisées (ectrosomie–hémimélie–atrophie des orteils, ectrodactylie), d'autres le sont peut-être, la boule œdémateuse ou hémorragique ayant disparu (absence de la phalange unguéale, syndactylie par accollement), mais le fait que 22,2 pour cent seulement des malformations des membres sont accompagnées d'hémorragies ou d'œdèmes localisés aux membres donne à penser que dans la majorité des cas la malformation est due à un arrêt de développement réalisé par un autre mécanisme. L'absence d'hémorragies localisées dans les malformations du bec montre qu'il en est de même pour elles.

En somme, il nous apparaît que chez l'embryon de Poule, seules quelques malformations des membres pourraient s'être réalisées d'après le mécanisme auquel Jost a donné le nom d'acroblapsie (*ακρος* situé à l'extrémité et *βλαπτω* nuire, léser) qui déterminerait une régression secondaire d'extrémités déjà bien ébauchées en opposition avec le mécanisme d'arrêt du développement plus ou moins précoce par lequel se réalisent d'ordinaire ces malformations.

Nous sommes ainsi amenés à reconnaître deux mécanismes différents pour la réalisation des malformations que nous avons obtenues. (1) Hémorragie ou œdème localisé; mais comme il n'est pas possible de dire dans chaque cas si l'hémorragie a détruit une ébauche déjà constituée, ou l'a empêché de se former, les termes de lymphoblapsie et d'hémoblapsie nous paraissent préférables à celui d'acroblapsie, puisqu'ils signifient seulement que l'œdème ou l'hémorragie localisée a joué un rôle dans l'apparition de la malformation. (2) Arrêt de développement analogue à celui qui donne les malformations obtenues après action de nombreux agents physiques et chimiques.

RÉSUMÉ

L'arrêt pendant 2 à 3 jours de l'incubation de l'œuf de Poule a un effet létal et un effet tératogène.

Cette technique détermine des malformations des paupières (ablépharie), des membres (ectromélie, hémimélie, syndactylie, ectrodactylie, polydactylie, atrophie des doigts et des orteils), et du bec (bec de lièvre, atrophie du bec) et des hémorragies et œdèmes localisés provoquant l'agénésie du duvet et le buphtalmos.

Le meilleur stade pour obtenir le maximum de chaque variété de malformation varie suivant la localisation de la malformation (refroidissement des œufs du quatrième au sixième jour pour le bec de lièvre, du cinquième au septième pour l'ablépharie, du sixième au huitième pour l'atrophie du bec et du septième au neuvième pour les membres).

SUMMARY

Interruption of incubation of the hen's egg for 2–3 days has a lethal effect and a teratogenic effect.

The treatment produces malformations of the eyelid (ablephary), of the limbs (ectromely, hemimely, syndactyly, ectrodactyly, polydactyly, atrophy of the fingers and toes), of the bill (hare-lip, atrophy), and haemorrhages and localized oedema which cause agenesis of down and buphthalmos.

The stage at which maximum numbers of each variety of malformation are obtained varies according to the localization of the malformation: cooling of the eggs between the fourth and the sixth day for hare-lip, between the fifth and seventh for ablephary, between the sixth and eighth for atrophy of the bill, and between the seventh and ninth for malformations of the limbs.

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EXPLICATION DES PLANCHES

Planche 1

Fig. 1. Ablépharie localisée à la partie postérieure des deux paupières. Absence du duvet entre l'œil et l'orifice auditif. Micrognathie inférieure. Mort 16 jours. Froid 5e à 7e jour.

Fig. 2. Ablépharie ne laissant subsister que la commissure antérieure avec un petit tubercule inférieur et un supérieur. Mort 22 jours. Froid 4e au 7e jour.

Fig. 3. Ablépharie totale sauf un petit tubercule antérieur. Absence du duvet dans la région supéro-interne. Le duvet a été enlevé sur une certaine zone qui apparaît piquetée. Bec de lièvre. Vivant 19 jours. Froid 4e au 6e jour.

Fig. 4. Ablépharie ne laissant subsister que la commissure postérieure. Atrophie du demi-bec inférieur. Vivant 18 jours. Froid 5e au 7e jour.

Fig. 5. Ablépharie localisée à la partie moyenne de la paupière inférieure. Micrognathie inférieure. Bec de lièvre. Vivant 17 jours. Froid 5e au 8e jour.

Fig. 6. Ablépharie localisée à la partie moyenne de la paupière supérieure avec buphtalmos. Vivant 18 jours. Froid du 4e au 7e jour.

Planche 2

Fig. 7. Ablépharie localisée à la partie postérieure de la paupière supérieure. Micrognathie supérieure. Vivant 19 jours. Froid du 4e au 6e jour.

Fig. 8. Atrophie des demi-becs supérieur et inférieur. Vivant 20 jours. Froid 6e au 8e jour.

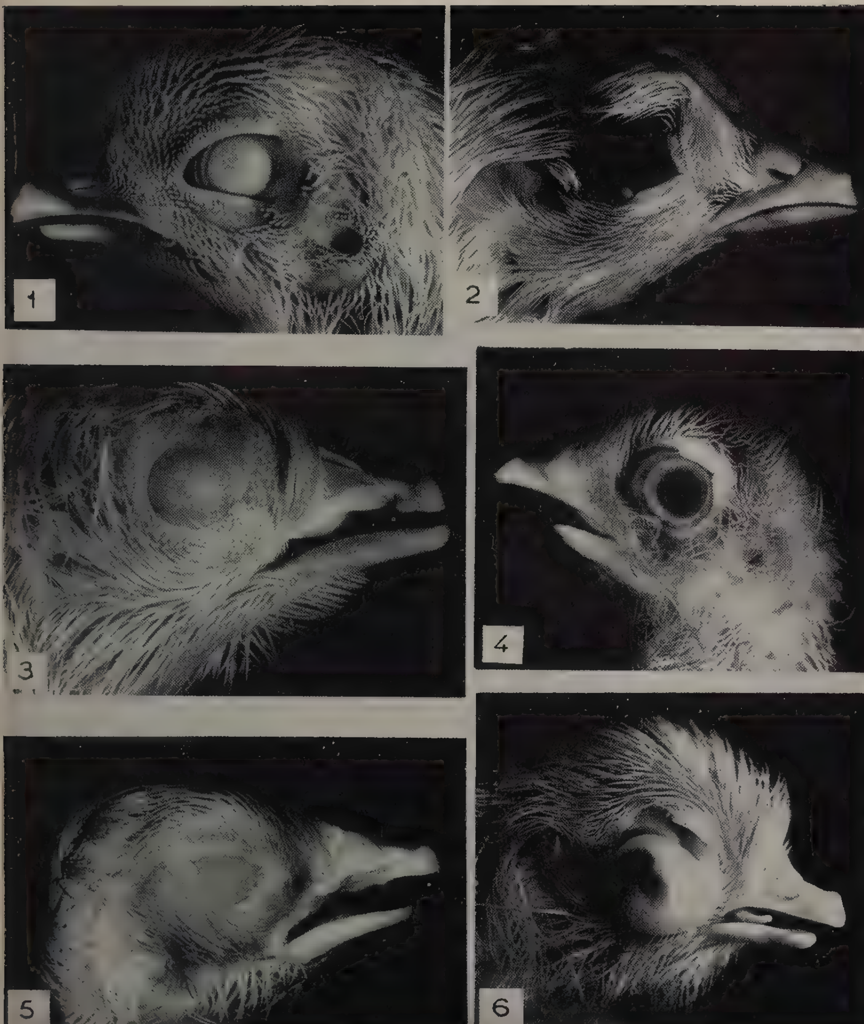

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Plate 1

P. ANCEL

Plate 2

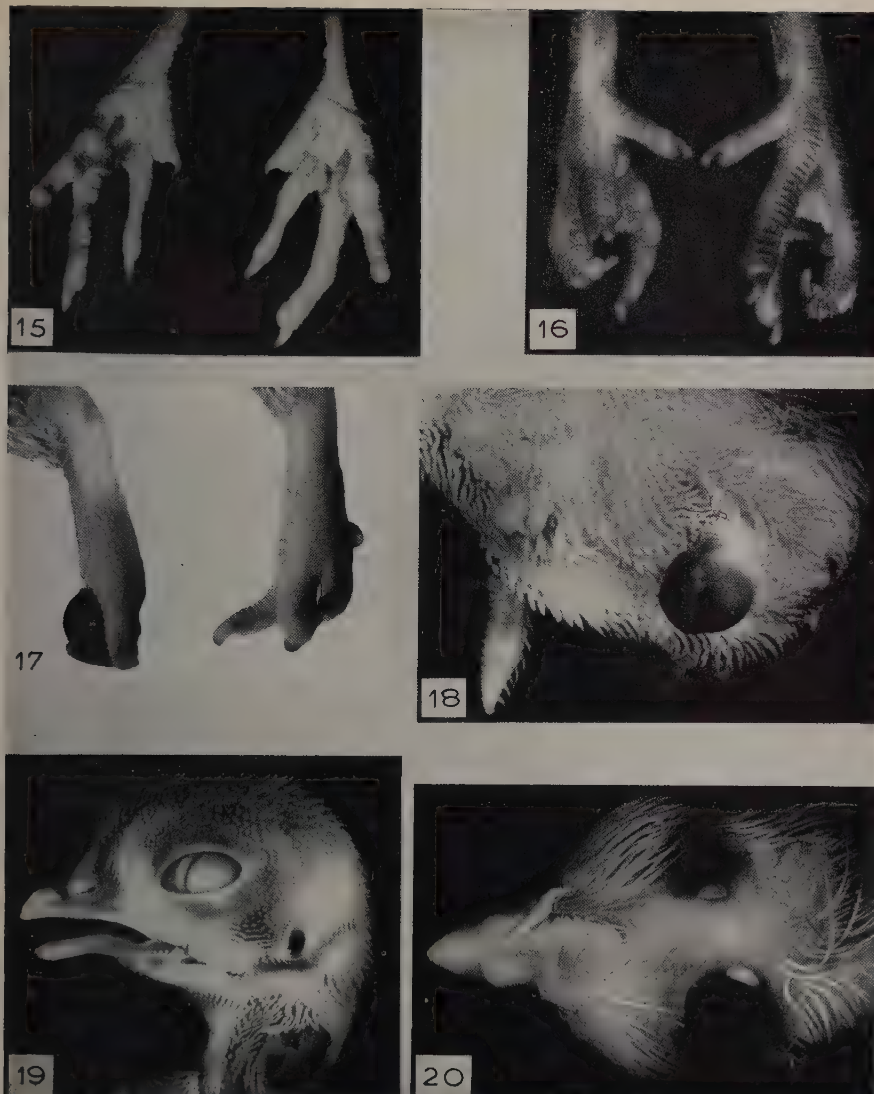

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Plate 3

FIG. 9. Atrophie du bec. Le demi-bec inférieur est formé par deux tiges cartilagineuses séparées par une membrane. Vivant 22 jours. Froid du 6e au 8e jour.

FIG. 10. Absence du premier orteil. Mort 23 jours. Froid du 5e au 7e jour.

FIG. 11. Atrophie des orteils et syndactylie avec membranes, partielle entre le 1er et le 2e orteil, totale pour les trois autres. Mort 19 jours. Froid 7e au 9e jour.

FIG. 12. Absence de la griffe du quatrième orteil. Vivant 18 jours. Froid du 7e au 9e jour.

FIG. 13. Atrophie du premier et absence du quatrième orteil. Mort 16 jours. Froid du 7e au 9e jour.

FIG. 14. Syndactylie par accolement des deux premiers orteils, y compris les griffes. Vivant 18 jours. Froid du 5e au 7e jour.

PLANCHE 3

FIG. 15. Malformation symétrique. Absence de la griffe du 4e orteil. Quatre jours après éclosion. Froid 7e au 9e jour.

FIG. 16. Dédoublement du deuxième orteil à droite. Déformation à angle droit de la phalange unguéale du troisième orteil des deux côtés. Mort 19 jours. Froid du 8e au 10e jour.

FIG. 17. Atrophie d'orteils porteurs d'une boule sanguine à leur extrémité, très volumineuse sur le 4e orteil à droite. Mort 16 jours. Froid 8e au 10e jour.

FIG. 18. Patte gauche remplacée par une grosse boule sanguine. Atrophie de l'aile gauche. Mort 16 jours. Froid 5e au 8e jour.

FIG. 19. Hémorragie localisée à la face latérale du cou. Mort 22 jours. Froid 7e au 9e jour.

FIG. 20. Tête d'un embryon vivant de 19 jours vue d'en haut. Symétriquement de chaque côté de la ligne médiane se trouve une grosse boule hémorragique et en dedans une petite boule d'œdème. Cas boules sont situées au-dessus et en arrière des yeux. Froid du 7e au 9e jour.

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Cell Mixtures of Different Species and Strains of Cellular Slime Moulds

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WITH ONE PLATE

INTRODUCTION

THE cellular slime moulds have, in their life history, a period of feeding of separate independent amoebae which subsequently stream together (by a process involving chemotaxis) to central collection points. Each of the resulting cell-masses differentiates into a fruiting body; in most species there is a delicate tapering celluose stalk which surrounds the large vacuolate stalk-cells, and an apical sorus containing a considerable quantity of individually encapsulated spore-cells.

There are a number of distinct and easily recognizable species and, moreover, it is a common observation (see Raper, 1951) that different isolates from the soil of any one species may have small but identifiable characteristics. It was found, for instance, in a previous study (Bonner & Shaw, 1957) that some strains under given environmental conditions formed a very long stalk, that is, delayed the process of spore differentiation, while others show rapid spore differentiation and correspondingly short stalks.

The purpose of this study was to examine the effect on the morphogenesis of these slime moulds of mixing the cells of different species and different strains in various ways. Such experiments show that there are varying degrees of compatibility among the combinations and that in many cases there are interesting types of compound fruiting bodies. Also it will be demonstrated that cells of a particular strain when disassociated tend to regroup within the cell-mass and certain parallels will be drawn between these results and those of sponge and other animal cell-dissociation experiments.

MATERIALS AND METHODS

The organisms were grown at 22° C. on plain, non-nutrient 2 per cent. agar upon which a loopful of *Escherichia coli* had been spread thin, a modification of a technique of Singh (1946) previously suggested to us by B. M. Shaffer. The experiments were run at room temperature (25° ±3° C.).

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The following species and strains were used:

Dictyostelium discoideum. This species is characterized by a stalkless migration period at the end of which stalk formation begins. The resulting fruiting body has a short stalk, a circular basal disk at the base of the stalk, and a white sorus. Only one strain of this species was used (Dd–1).

D. mucoroides. This species begins stalk formation at the end of aggregation and continues to build stalk throughout its migration period. Of the four strains used, Dm–2 invariably has a short migration phase, while Dm–4, Dm–11, and Dm–16 all have long migration under the conditions used. The latter three strains have minor morphological differences (e.g. Dm–4 has a serpentine rather than a straight stalk during migration) but these differences are immaterial to the present study.

D. lacteum. Similar to *D. mucoroides* except that it is very small, has virtually no migration, and its spores are round instead of capsule-shaped. Only one strain was used (D1–1 from K. B. Raper).

D. purpureum. The same as *D. mucoroides* except that the final sorus is purple in colour. Only one strain was used (Dp–2) which possesses the character of long migration.

Polysphondylium pallidum. During the beginning of its migration this species resembles *D. mucoroides*, although eventually it pinches off a series of cell-masses on the stalk, each one of which produces a group of small fruiting bodies coming off the main stalk as branches in a whorl. There is also one large terminal sorus which is white, as are all the secondary sori. Only one strain was used (Pp–2).

P. violaceum. The same as *P. pallidum* except that the sori are purple. Two strains were used, of which Pv–1 has slightly shorter migration than Pv–2.

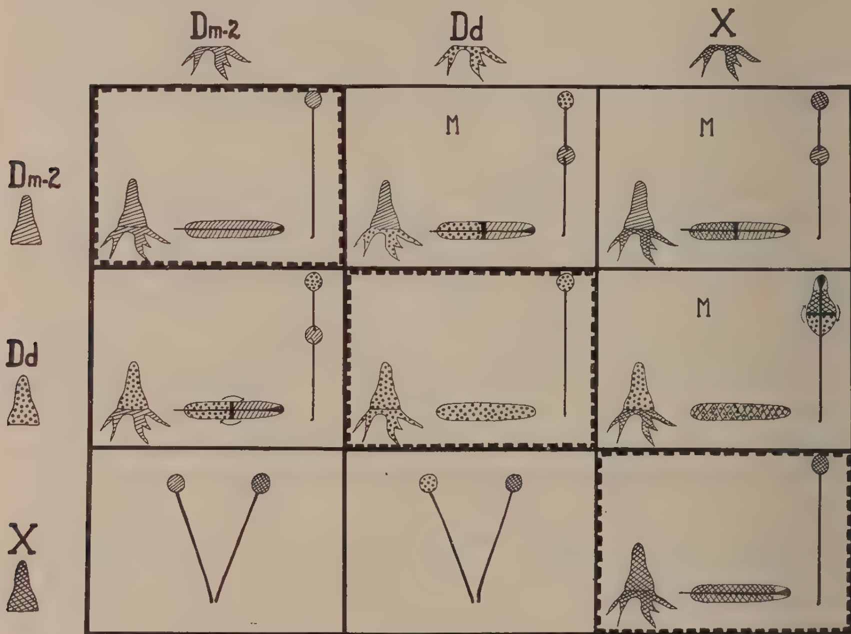
RESULTS

Grafts in the aggregation stage

The cells of these species and strains were mixed in three different ways. In the majority of the experiments the following procedure was used. First the centre of an aggregation pattern of one strain was removed with a hair loop. Then the centre of an aggregation pattern of another strain was placed in the middle of this aggregate. If there was any degree of compatibility at all the aggregating streams would join the foreign cell-mass, so that presumably approximately the anterior half was one strain and the posterior half another.

For the sake of simplicity of presentation it may be useful to the reader to follow this discussion by referring to Text-fig. 1. As indicated in the margins of this diagram, there are three groups of strains that have discrete reactions: (1) Dm–2; (2) Dd–1; (3) Dm–4, Dm–11, Dm–16, Dp–2, all of which for convenience are designated *X*. If, now, a Dm–2 anterior end is grafted on to another Dm–2 posterior aggregation stream, then the result (Text-fig. 1, upper left box), will be a normal complete Dm–2 fruiting body. Similarly, Dd grafted to Dd will give

a normal Dd (central box), and an *X* grafted to an *X* will give a normal *X* (lower right-hand box). In this latter case this is only true if the *X* is the same strain in both anterior and posterior portions (e.g. Dm–16 grafted on to Dm–16), for any other combinations of *X* (e.g. Dm–16 grafted on to Dm–11) invariably result in a complete separation of the cells to form two distinct fruiting bodies, showing no tendency to merge. This point will be touched upon again later.



TEXT-FIG. 1. A diagram illustrating the results of the grafts made during the aggregation stage. The centre of one strain (indicated on the left) is placed on the decapitated aggregation pattern of another strain (indicated on the top). If the graft involves the same strain from both parts the result is normal development (indicated by the three squares with serrated borders). *M* designates those combinations which were also tested by mixing the cells at the migration stage. For further details see the text.

Dm-2 in front of Dd. If the anterior end of Dm–2, which is a short migration form, is grafted on to the posterior portion of Dd, a form which possesses the power of prolonged migration, then the two cell-masses will stay fused, but the final result, in all 8 cases tried, is a double fruiting body (Text-fig. 1, centre top box). It could readily be observed that the first fruiting body to form was the anterior Dm–2 (which exhibits short migration and rapid spore differentiation), and that the previously posterior Dd pushes past the final spores of the Dm–2 and either forms a fruiting body directly on top of the sorus of the Dm–2 (as shown in the diagram), or migrates away on to the agar substratum and fruits

elsewhere. The fruiting bodies of the two species are sufficiently distinct, so that following the separate strains from the merged mass is relatively easy. More detailed studies, including the use of paraffin sections, were made on the next combination, the results of which are essentially similar.

Dm–2 in front of X. This combination (Text-fig. 1, upper right box) again uses the Dm–2 tips, but in this case the aggregating patterns of the various members of the *X* complex were placed in the posterior ends. The reason, as stated above, for the use of this *X* grouping is that all members of the group behave the same way (31 cases with Dm–4, 4 with Dm–11, 6 with Dm–16, 4 with Dp–2).

The fact that the anterior, rapidly differentiating Dm–2 formed a fruiting body first, and the *X* pushed through to form the 'second story' fruiting body, was seen especially strikingly when Dp–2 was used for the *X*. Since this species has a purple sorus, the lower sorus was pure white and the upper sorus purple, clearly indicating that the posterior Dp–2 had passed through the Dm–2 cell-mass. This was further tested by taking the spores from the upper and lower sori, culturing them, and examining the progeny, and there was no evidence of intermingling of cell-types.

The mechanism of this 'passing through' was then examined by the use of paraffin sections. As was known from a previous study, the periodic acid-Schiff technique is especially useful at revealing the state of differentiation of a migrating mass. (For the details of the method see Bonner, Chiquoine, & Kolderie, 1955.) Sections were prepared of the Dm–2 to *X* grafts at various stages and, as shown in the Plate, fig. A_1, there is a sharp, discrete division line between the two strains. The *X* strain shows no differentiation whatsoever until the Dm–2 undergoes its final differentiation. Then, as shown in the Plate, fig. A_2, the *X* shows pre-spore and pre-stalk zones as well as the beginning of stalk formation at the tip. It quickly pushes past the differentiated spores of the Dm–2, using the sorus of Dm–2 as a platform. The stalks of the two strains never join; the tip of the Dm–2 stalk will lie near the base of the *X* stalk.

Dd in front of Dm–2. Another combination possible is to put Dd tips in the centre of Dm–2 aggregation streams (Text-fig. 1, middle, left box), and this, it will be noted, is the reverse of the first experiment. Here the rapidly differentiating Dm–2 is initially behind the long migration Dd, yet the end result is the same as before, namely, the Dm–2 produces its fruiting body on the agar, and the Dd again has the upper, 'second story' position. (This was true in 15 cases, in 11 others the separation was complete from the beginning.) In a number of the cases the spores were removed from the two sori and their progeny tested, which completely confirmed the apparent composition of the two fruiting bodies.

In some of the cases, for an interval following aggregation, there was a stalk-less migration which is characteristic of the anteriorly placed Dd. In other cases stalk formation occurred at the very beginning and gave the general appearance of Dm–2. This puzzling behaviour was again analysed by making stained

sections, and it was found that fairly rapidly following the end of aggregation there is a redistribution of the strains; in some way the Dm–2 cells assume a more anterior position. There is a suggestion from the slides (Plate, fig. B) that this occurred by small groups of Dm–2 cells passing through the Dd cells. There is no doubt, however, that the Dm–2 cells did become anterior, with the result that they formed the first lower fruiting body, and the Dd climbed past them to build the upper fruiting body.

Dd in front of X. Placing Dd centres into the aggregation streams of *X* gave a more complete combining of the two strains, when the experiment was successful. In 35 cases, 14 showed complete separation and the remaining 21 gave a single fruiting body. If spores were tested from the single sorus of one of these combined fruiting bodies they gave both parental types. Occasionally the spores of one of the two would differentiate sooner and be left behind on the stalk, thus superficially giving the appearance of a double fruiting body, though in fact this was only a single stalk with a ring of spores about its middle.

In the stained sections of this combination there was no sharp division line between the two strains during the migration period; the cells appeared to intermingle. The migration was characteristic of Dd, showing no stalk formation. As the final spore differentiation approached it was obvious, however, that the pre-spores of each strain had sorted out into two cohesive, compact cell-masses, one lying adjacent to the other (Plate, fig. C). There was no consistency as to the sequence, for in some cases the Dd pre-spore mass was anterior, and in others it was the *X* pre-spore mass. There is no way at present of ascertaining whether one or both of the strains contributed cells in the formation of the stalk.

Dd in front of Dd. Some of the above experiments were done with Dd stained with vital Nile blue sulphate (Bonner, 1952), and it was noticed that when a blue tip was grafted on to a white aggregation stream of another strain there was a rapid mixing of the blue, so that the whole sausage-shaped cell-mass was blue by the beginning of migration. To see whether this rapid mixing normally occurred within any one strain, the same experiment was repeated by grafting blue tips of Dd on to colourless aggregation streams of Dd and vice versa. Invariably the resulting cell-mass would be uniformly blue, indicating a violent mixing of cells at the end of aggregation. This result is surprising, since it is well known that when the graft is made later, during the migration stage, the division line remains fairly constant for a number of hours (Raper, 1940, 1941; Bonner, 1952, 1957). Apparently the end of aggregation is a critical period of cell distribution and should be investigated further for its role in morphogenesis.

All other combinations. A number of other combinations were tried, and in every case there was a complete separation of the two strains to form two fruiting bodies. In Text-fig. 1, *X* grafted on to Dm–2 (23 cases) and *X* grafted on to Dd (18 cases) gave such a result. As mentioned before, all the members of the *X* complex (Dm–4, Dm–11, Dm–16, Dp–2) were tried against each other in reciprocal crosses with no mergers. Also *Dictyostelium lacteum* was combined with Dd,

Dm–2, Dm–4, and *Polysphondylium violaceum* (Pv–2). Finally, Pv–1, Pv–2, and *P. pallidum* (Pp–2) were combined in various ways with the same lack of unification of the cells.

Cell mixtures at the migration stage

The second of the three methods used to combine the strains was to take two young migrating masses, each from a different strain, lop off the anterior $\frac{1}{10}$ of each, and thoroughly mix the two with an eyelash attached to a glass tube so that there was one small compact circular blob of mixed cells. The mixtures made are indicated by the letter *M* in Text-fig. 1.

Dm–2 and Dd mixtures. Mixing appeared to give an even closer association than did grafting, for in 7 cases out of 10 there arose a single fruiting body which contained the spores of both strains (the remaining 3 cases were too confused to interpret). Fig. D of the Plate shows a stained section of such a combined fruiting body, and it is obvious that the cells of each strain have regrouped into discrete, though cohesive, masses of pre-spores. There was no stalkless migration characteristic of Dd.

Dm–2 and X mixtures. With this combination the result was identical with the grafting experiments, there was a sorting out to the extent that a double fruiting body was produced in which the lower sorus was Dm–2 and the upper sorus was *X* (which was demonstrated by replating of the spores (11 cases)).

Dd and X mixtures. As with the grafts, a single fruiting body was produced in which the cells of each strain regrouped themselves within one cohesive mass of spore-cells (10 cases). It should be noted here that this experiment is identical with that of Raper & Thom (1941), who mixed migrating masses of *D. discoideum* and *D. purpureum*, obtaining fruiting bodies made up of the cells of both species.

X and X mixtures. With one exception, no other mixture fused in any permanent way and separate fruiting bodies would result. In one case out of 7 (Dm–16, Dp–2 mixtures) there was a clearcut double fruiting body in which Dm–16 formed the lower one, and Dp–2 the upper one.

Grafts at the migration stage

A few grafts were made at the migration stage by grafting the anterior half of the migrating mass of one strain on to the posterior half of another. This was tried with Dm–2 grafted on to Dd (2 cases) and *X* (4 cases) and, as with the aggregation grafts, they gave rise to double fruiting bodies. The only point of interest here is that when Dd was placed in front of Dm–2 they separated completely (3 cases). It will be remembered that with the aggregation grafts (Text-fig. 1, left, middle box) the Dm–2 crawled ahead of the Dd to assume an anterior position, and that from the vital blue Dd's grafted on to colourless aggregation streams of Dd there was evidence of violent cell redistribution at the end of aggregation. Therefore this separation of Dm–2 and Dd at the migration stage is

consistent, for the grafting has been made after the period of active cell redistribution, and there is no longer any opportunity for the Dm–2 cells to surge forward and intermingle with the cells of the Dd.

DISCUSSION

The mixing of cells of different species, particularly at the feeding or vegetative stage, was first performed many years ago by Olive (1902). He says: '. . . two well marked species of *Dictyostelium*, one for example with white spores and another with dark, sown in the same spot of a nutrient agar tube, will result in fructification showing the two distinct forms growing side by side without any trace of intermixture.' This matter was thoroughly investigated by Raper & Thom (1941), who not only confirmed Olive but greatly extended the observations. They showed that mixed spores or vegetative amoebae of *D. discoideum* and *P. violaceum* gave separate aggregations, while mixtures of *D. discoideum* and *D. mucoroides* formed common ones, but separate fruiting bodies were formed at the centre. Grafts during the migration stage displayed some temporary coalescence in the combination of *D. discoideum* and *D. purpureum*. Furthermore, they found that these same two, as mentioned above, would form a unified fruiting body when their migrating cell-masses were thoroughly intermixed.

Gregg (1956) has pursued this matter of adhesion between cells by injecting amoebae of different species into rabbits and obtaining 'anti-amoeba' antibodies. He found that the antibodies were species specific with vegetative amoebae, but that this was not the case once the aggregation stage was reached, and on the basis of these experiments he suggests that a general tendency for surface adhesion appears at the aggregation stage, thereby possibly explaining the observation of Raper & Thom (1941) that the combining of *D. discoideum* and *D. purpureum* cells can only take place at later stages of development. It is, of course, difficult to know to what extent Gregg's experiments are revealing properties of the cell surface, although there is no doubt, as Shaffer (1957 *a*, *b*) has shown in a series of most interesting observations, that the stickiness of cells, along with specific differences in the acrasin (or chemotactic substance) of different species, plays an important role in the aggregation and cell association phases. Furthermore, he has shown that certain combinations of species adhere more readily to one another than others.

One of the curious facts that comes from our experiments is that specific differences between strains of one species are as great, or even greater, than those between different species. Certain strains of *D. mucoroides* were completely unable to permanently coalesce with one another, and this was true as well of the two strains of *P. violaceum*. Yet partial merging was possible between *D. discoideum* and particular strains of *D. mucoroides* and *D. purpureum*. Each strain represents a separate isolate from nature, and M. F. Filosa from our

laboratory is now in the process of further analysing the nature of these strains and their genetic constitution.

The results obtained upon mixing strains are obviously not merely a matter of surface specificities. This is particularly evident in the case of Dm–2, where in part its unique properties may be considered in terms of its rate of differentiation. The fact that in this strain spore differentiation occurs so quickly means that it will supply, in a short time, a base for the second fruiting body. In other words, the basis of the double fruiting body formation is the differential between the rate of spore formation in Dm–2 and its partner (Dd or all the strains that comprise *X*). It is especially interesting that the rate of differentiation of two groups of cells lying side by side (e.g. Plate, fig. A) can be so completely independent; it indicates that this rate is intrinsically controlled within the cells.

Another noteworthy point is that when Dm–2 leads the cell-mass (Plate, fig. A_1) the posterior strain remains totally undifferentiated for an abnormally long period; histologically it appears to be in an aggregating condition despite the fact that it may have been part of a migrating or culminating mass for some hours. It only regains its ability to differentiate after the Dm–2 has completely sporulated. This is clear evidence that the anterior Dm–2 holds back the development of the other strain.

If we now examine the interesting property of the sorting out of cells within a cell mixture, it is possible to classify the results into three degrees of the extent to which the cells of two strains pull away from one another—three degrees of cell compatibility. (1) At one extreme the cells separate completely to form two separate fruiting bodies. (2) In the intermediate case double fruiting bodies are formed, which is characteristic when Dm–2 is present. (3) The highest degree of compatibility found thus far is when a single sorus contains two separate, cohesive patches of spores, each belonging to one of the strains. Since the cells in this case do still pull apart and regroup there is obviously some incompatibility, and theoretically it should be possible to find two strains that are completely compatible in which the spores of both will spread at random through one common sorus.

This matter of sorting out is now a well-recognized phenomenon in experiments with dissociated animal cells. This is no place for a detailed history of the discovery of the phenomenon, but the original idea stems from the work of H. V. Wilson (1907), who pushed sponges through bolting cloth and noted that the dissociated cells reorganized to form a new functional sponge. At the time of his original experiments he made the incorrect assumption that the differentiated cells have reverted back to some embryonic type and then re-differentiated following coalescence. This error was first corrected by J. S. Huxley (1911, 1921), who worked with another species of sponge and showed that the different cells retained their differentiation following dissociation. He pointed out that Driesch's dictum, that the fate of a cell is the function of its position, does not hold in this case, but the reverse, for the position of a cell is a function of its

differentiation, that is the differentiated cells wander about in the coalesced clump of dissociated cells until they find their proper location. This point has been confirmed in numerous ways by different workers, all of which is excellently reviewed by P. Brien (1937), who has contributed some evidence himself.

Much the same story holds for the reassociation of coelenterate cells, and more recently there have been some remarkably convincing experiments on dissociated vertebrate cells. In 1952 Weiss & Andres injected dissociated presumptive melanoblasts into the blood-stream of chick embryos and they found that these cells become lodged in their appropriate region in the embryo. Townes & Holtfreter (1955) were able to show such specific reorganization in dissociated amphibian embryos, although the best evidence that the cells retain their differentiations comes from recent work. In particular, Trinkaus (1957) has been able to follow the cell types using isotope markers, and Moscona (1957) has used an elegant method with a mixture of cells from different species. Previously he had developed a method of dissociating cells by the use of trypsin and then to mark his cells he used combinations of chick and mouse cells, each of which is histologically recognizable. If mouse and chick cartilage were mixed, a mass of continuous cartilage resulted in which mouse and chick cartilage-cells were randomly distributed. However, if mouse cartilage-cells were mixed with chick kidney-cells then the cells by migration formed discrete groups of a mass of cartilage and a mass of kidney-tissue. The important proof is that all the cartilage was mouse and all the kidney was chick; there had been no cell transformation but merely a regrouping of the cells.

It should be noted that these experiments of Moscona differ from the slime-mould experiments reported here, in that his cells showed no specificity in their regrouping with respect to species, but only with respect to the tissue. In the slime moulds, the cells show species specificities in their regrouping, but thus far we have not demonstrated any tissue regroupings. If we are to draw a conclusion from this paradox it is perhaps that the common denominator in this interesting process of sorting out or regrouping is not the degree of differentiation, or determination, of the cells, but rather the degree of surface compatibility among the cells. Cells which are completely compatible will mix randomly, cells which are completely incompatible will separate completely, cells which show an intermediate degree of surface compatibility will regroup by sorting out. This intermediate degree of surface compatibility may be produced either by species differences, as in the slime moulds, or differences in cell differentiation, as in vertebrate cells. Moreover, in the case of sponges this incompatibility can be produced both ways, for Galtsoff (1929) showed that in mixtures of cells of different sponges there was a sorting out with respect to differentiation as well as a sorting out with respect to species.

SUMMARY

The cells of various species and strains of cellular slime moulds have been mixed by making grafts at the aggregation stage and by thoroughly mixing the cells at the migration stage. Depending upon the strains used, varying degrees of cell compatibility or adhesion between the cells was observed. There was either (1) a complete separation of the strains; (2) a partial merger to form a double fruiting body, one standing on the sorus of the other; (3) a single fruiting body on which the pre-spores of both strains regrouped to form two cohesive blocks of cells within one sorus.

This regrouping phenomenon is also characteristic of many dissociated animal cells where partially or completely differentiated cells will regroup with their kind. It is suggested that the basis of this regrouping phenomenon is the result of an intermediate degree of surface compatibility—not so great as to produce a complete separation of the cells, and not so little as to permit a random intermingling of the cells.

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EXPLANATION OF PLATE

Photographs of sections stained by the periodic acid-Schiff method for non-starch polysaccharides. The text should be consulted for details.

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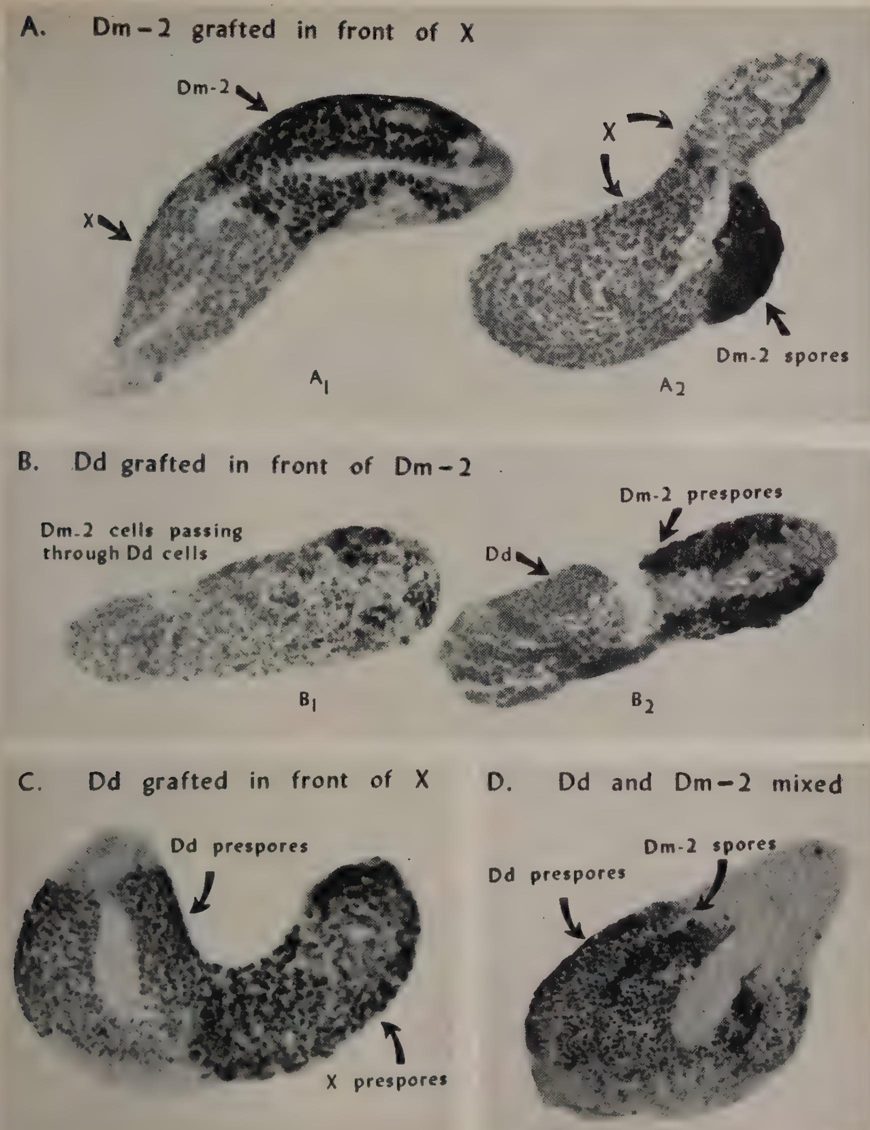

J. T. BONNER *and* M. S. ADAMS

Action of Triethanomelamine (TEM) on Early Stages of Chick Embryos

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WITH TWO PLATES

OF many closely related compounds investigated recently from the point of view of cytotoxity and tumour-inhibiting properties 2,4,6-tri-(ethyleneimino)-1,3,5-triazine (called also triethanomelamine or TEM) proved to be the most effective substance against the Walker rat carcinoma. First descriptions of this fact were published in 1950 (Buckley *et al.* and Burchenal *et al.*). Also in 1950 appeared the publication of the first results of the clinical application of TEM at the First International Cancer Congress (Mueller & Miller). Later on Karnovsky *et al.* (1951) and Petersen *et al.* (1951) recommended procedures for parenteral and oral administration of this substance in clinical treatment. Very recently TEM has been introduced in a commercial form for oral and parenteral administration in cases of leukaemia, Hodgkin's disease, polycythaemia vera, and other neoplastic conditions.

The cytotoxic activity of TEM is very similar to that of the nitrogen mustards (Philips & Thiersch, 1950), chromosome breakage with simultaneous chromosomal rearrangements, chromosome bridges and other malformations being observed by Fahmy & Fahmy (1953, 1954, 1955), Lüers (1953), and Herskowitz (1955). In somewhat higher doses TEM appears to act as an antimitotic agent (Hendry *et al.*, 1951).

The present work was undertaken to investigate the influence of TEM on early stages of the embryonic development of chick embryos and on the formation of the first anlagen of some organs.

MATERIAL AND METHODS

In the first part of this work a solution of TEM in 0·9 per cent. NaCl was injected into the albumen of fertilized chicken eggs, which had been incubated at 37·5° C. for 22 hours until the stage of the primitive streak was reached. The puncture in the shell was closed with wax and the eggs left for approximately 20 hours to allow the injected solution to diffuse throughout the albumen. The eggs were afterwards reincubated for another 24 hours, the embryos taken out, rinsed in saline solution, and fixed. The whole period of the incubation *in ovo*

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was consequently about 46 hours with a break of about 24 hours for injection and diffusion.

In the second part of this work we used chick embryos cultivated *in vitro* according to the method described by New (1955). The fertilized eggs were incubated for 22–23 hours, then the embryos transferred to watch-glasses; those which were in the stage of the primitive streak were selected and treated with TEM or left as controls. TEM was applied in solution in the liquid egg albumen put round the plastic ring used for this method. The embryos were then incubated *in vitro* for different periods of time, usually about 24 hours, but always till the controls reached the stage of 12–16 somites.

In the experiments in the first part of this work, after preliminary tests, three concentrations of TEM were used: 10^{-5}, 2×10^{-5}, and 5×10^{-5}. Embryos cultivated *in vitro* were treated with lower doses: 2×10^{-6}, 3×10^{-6}, and 5×10^{-6}. Higher concentrations were used *in ovo* because of the additional dilution of TEM by the albumen.

For the comparison of the developmental stages the works of Patten (1950) and Hamburger & Hamilton (1951) were used.

The embryos were examined histologically and cytologically. For histological purposes the embryos were fixed in alcoholic Bouin solution, embedded in wax, and after sectioning stained with haematoxylin and eosin. For cytological purposes the embryos were fixed in Carnoy's fluid and stained with methyl green pyronin. The whole mounts were stained with Mayer's haematoxylin.

The TEM used was supplied by Imperial Chemical (Pharmaceuticals) Ltd., Manchester.

RESULTS

In both groups of experiments, i.e. whether TEM was applied *in ovo* or *in vitro*, the results were very similar and the intensity of changes depended roughly on the concentration used. However, there was a considerable variation in response between embryos treated with the same concentrations, this variation being much greater between embryos treated *in ovo* than between embryos cultivated *in vitro*.

Macroscopical changes

The lowest concentrations, 10^{-5} and 2×10^{-6} *in ovo* and *in vitro* respectively, caused a distinct retardation of the growth and development of embryos. Whereas the control embryos injected with saline, or treated with the corresponding mixture of the liquid albumen and saline solution, were normally in the stage of 12–16 somites by the end of the experiment, the experimental embryos had only 6–9 somites. Many of the experimental embryos appeared to have peculiar hollows inside the somites, more particularly in the posterior ones, though in some embryos many somites were damaged. The embryos in this group also showed some changes in the head, which was considerably widened,

probably due to increased accumulation of the head mesenchyme cells, whereas the development of the brain appeared to be slightly retarded (Plate 1, compare figs. 1 and 2).

The intermediate concentrations, 2×10^{-5} and 3×10^{-6} respectively, caused more distinct changes in development. The retardation was stronger, often with almost complete atrophy or considerable under-development of the somites. The neural tube, whether closed or not, was almost normal in appearance and shape. In the head there was usually much less mesenchyme than in embryos given the lower concentrations (Plate 1, fig. 3).

The strongest concentrations (5×10^{-5} or 5×10^{-6}) caused also strong retardation of development. The somites were often completely atrophied. In some embryos vesicles were found on both sides of the axis (Plate 1, fig. 4). They were usually located a short distance away from the axis, not right against it as is typical for the vesicles seen in embryos treated with purine analogues (Waddington, Feldman, & Perry, 1955).

Microscopic changes

According to histological observations it appears that the influence of TEM on these embryos is directed mainly to the mesodermal organs, primarily against somites. In fig. 5 (Plate 1) is shown a transverse section of the 8th pair of somites of a control embryo. In fig. 6 is a similar section of the 5th pair of an experimental embryo treated with TEM at a concentration of 10^{-5} injected *in ovo*. Comparison shows that the development in this case was nearly the same, but the somites of the experimental embryo are empty with an absence of the core of the somites, which normally consists of irregularly arranged cells.

After treatment of embryos *in vitro* with TEM of 3×10^{-6} concentration or *in ovo* with a concentration of 2×10^{-5} the somites of some embryos were further damaged. In some cases there was an extreme retardation of development with very marked effects on the somites, which appeared to be completely hollow (Plate 1, figs. 7 and 8). In other cases the cells of the somites have been more or less separated, and the whole organ to some extent disintegrated, the somitic cells becoming more like mesenchymal cells. In such embryos changes could be found in other tissues and organs, for instance in the neural tube and notochord, though these seem to be more resistant to TEM than the somites (Plate 2, fig. 9).

Many of the embryos in the second and third group, i.e. those treated with TEM *in ovo* using concentrations of 2×10^{-5} or 5×10^{-5} and *in vitro* using concentrations of 3×10^{-6} or 5×10^{-6}, appeared to have very large vacuoles or bubbles lying along both sides of the central organs of the embryo. In the transverse sections of such embryos a distinct disintegration of the somites and a marked widening of the coelom could be seen. These widenings were visible in the whole mounts as vesicles, which appeared to be present not only between

both somato- and splanchnopleure, but also between splanchnopleure and the entoderm layer (Plate 2, fig. 10). The vesicles were thus of quite a different kind to those resulting from purine analogues, which are due to the disintegration of the mesoderm being formed from the primitive streak (Waddington *et al.*, 1955).

The changes in the amount of the head mesenchyme caused by the lowest doses of TEM usually appeared distinctly, but did not occur in all the embryos examined. A moderate accumulation of mesenchyme cells is seen in fig. 11 (Plate 2) in comparison with the control head of fig. 12.

After treatment with any of the concentrations of TEM, both *in ovo* and *in vitro*, pronounced changes in nuclei and nucleoli of cells of the somites, neural tube, and notochord could be seen. By staining with methyl green pyronin after fixation with Carnoy's fluid the nuclei of experimental embryos appeared to be nearly three times larger than those of the controls, the nucleoli showing still greater hypertrophy, and the cells were as a whole hyperplastically changed (Plate 2, figs. 13 and 14).

DISCUSSION

2,4,6-Tri-(ethyleneimino)-1,3,5-triazine is an extremely toxic compound, as can be seen from the concentrations used in the experiments in this and other works. In medicine it is being increasingly used against leukaemia, Hodgkin's disease, polycythemia vera, i.e. against neoplastic conditions of particular organs of mesodermal origin. It has a very toxic effect on the bone marrow, where it affects the haemopoetic activity selectively (Gellhorn, Klingerman, & Jaffe, 1952). In stronger doses it produces rapid and widespread damage in lymphoid tissues (Hendry *et al.*, 1951). The bone marrow and lymphatic glands are typical organs of mesodermal origin and this seems congruent with the rather selective activity of TEM in relation to the somites shown in this work. However, in amphibian embryos it is not the mesoderm but the neural tissue which is most sensitive to the drug (Waddington, 1958). Presumably this sensitivity depends on the precise chemical constitution of the cells; but since the mode of action of TEM on biological materials is not fully understood, it is not clear precisely what the relevant factors are.

Enlargement of the nuclei and nucleoli are frequent effects of the action of cytotoxic agents; after treatment with TEM these changes have been observed mostly in the tissues of the somites and the neural tube. They are presumably a consequence of the inhibition of mitosis. Similar enlargements of nucleoli and the production of giant cells were observed in a quite different material, planaria, in the process of regeneration after treatment with TEM (Pedersen, 1957), and also in amphibian embryos by Töndury (1955).

SUMMARY

1. Triethanomelamine (TEM), applied to chick embryos in the stage of the primitive streak both *in ovo* and *in vitro*, produces retardation of development which is the greater the higher the dose used.

2. The embryonic organs most sensitive to TEM appeared to be the somites, which become empty through the disappearance of the central core.

3. In higher doses the somites undergo more or less complete disintegration, the somitic cells being separated and resembling mesenchyme cells.

4. TEM causes a considerable enlargement and widening of the coelom and of the space between splanchnopleure and the entoderm layer.

5. After treatment by lower doses there appears a slight accumulation of the head mesenchyme cells.

6. TEM causes a considerable enlargement of the cells, nuclei and nucleoli of the somitic, neural tube, and notochord cells.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Macroscopical view of a control chick embryo of 13 somite stage. ×20.

FIG. 2. Macroscopical view of a chick embryo treated *in ovo* with 0·5 ml. solution of TEM (concentration 10^{-5}). ×20.

FIG. 3. Macroscopical view of a chick embryo treated *in ovo* with 0·5 ml. solution of TEM (concentration 2×10^{-5}). ×20.

FIG. 4. Pronounced changes after injection into the egg of 0·5 ml. solution of TEM (concentration 5×10^{-5}). ×20.

FIG. 5. Transverse section of a control chick embryo through the 8th pair of somites. ×280.

FIG. 6. Transverse section through the 5th pair of somites of a chick embryo treated *in ovo* with 0·5 c.c. solution of TEM (concentration 10^{-5}). ×280.

FIG. 7. Transverse section through somites of a chick embryo treated *in ovo* with 0·5 c.c. solution of TEM (concentration 2×10^{-5}). ×280.

FIG. 8. Transverse section through somites of a chick embryo treated *in vitro* with solution of TEM (concentration 3×10^{-6}). ×280.

PLATE 2

FIG. 9. Transverse section of somites of a chick embryo treated *in vitro* with solution of TEM (concentration 3×10^{-6}). ×280.

FIG. 10. Transverse section of a chick embryo after treatment with solution of TEM *in ovo* (concentration 5×10^{-5}). On the left of the neural tube there are vesicles between somatopleure and splanchnopleure and between splanchnopleure and endoderm. ×110.

FIG. 11. Transverse section of the head of a chick embryo treated with TEM *in ovo* (concentration 10^{-5}). ×100.

FIG. 12. Transverse section of the head of a control chick embryo at 13 somite stage. ×100.

FIG. 13. The cells of the neural tube of a control chick embryo at 13 somite stage. ×700.

FIG. 14. The cells of the neural tube of a chick embryo after treatment with TEM *in vitro* (concentration 3×10^{-6}). ×700.

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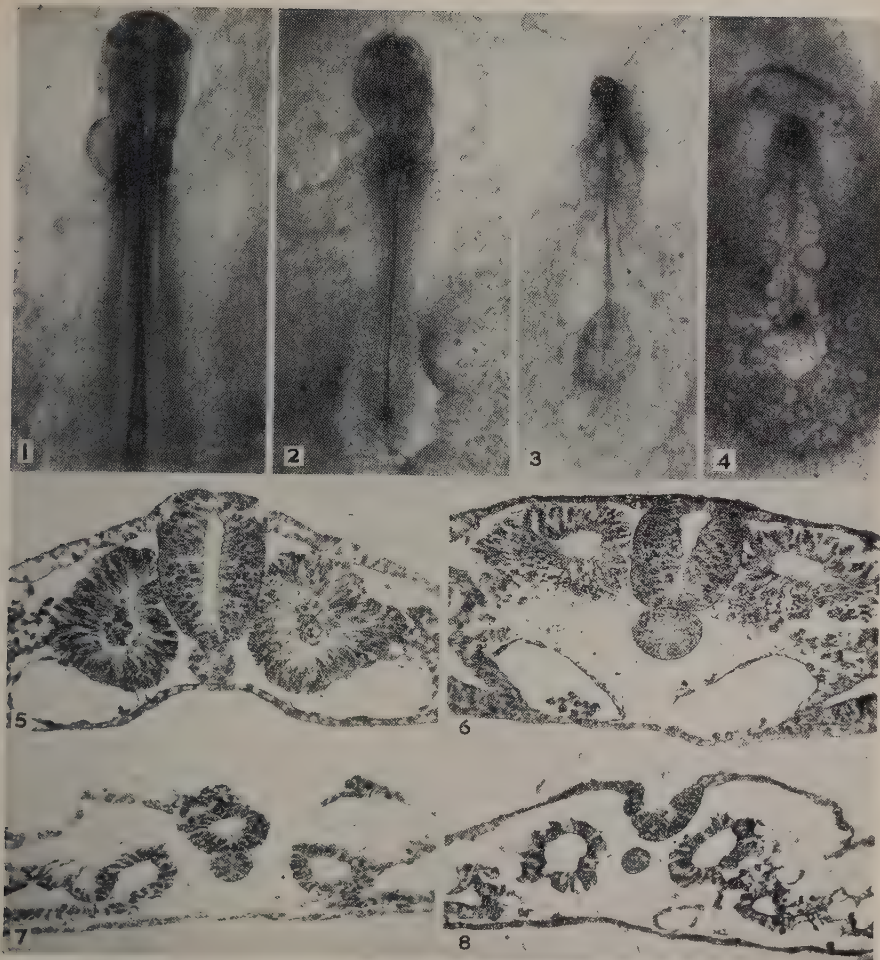
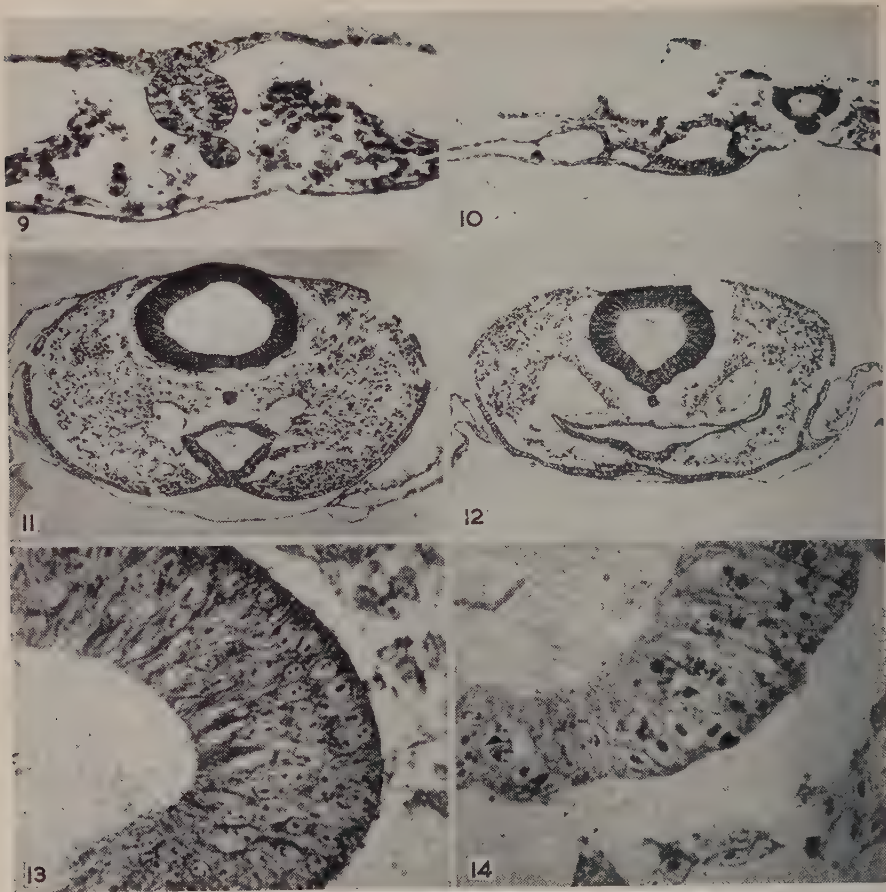


A. JURAND

Plate 1



A. JURAND

Plate 2

A Note on the Effects of some Cytotoxic Substances on Amphibian Embryos

by C. H. WADDINGTON[1]
From the Institute of Animal Genetics, Edinburgh

WITH ONE PLATE

In connexion with the experiments of Jurand (1958) on the action of TEM on avian embryos, it seems worthwhile describing very shortly some observations on the effects of this substance, and also of Myleran, on early amphibian embryos. Bodenstein (1954 and earlier) has described some of the cytological results of the administration of various 'radio-mimetic' substances to amphibian embryos, including nitrogen mustards and TEM. The effects on the development of the eye were particularly considered, and they have also been discussed and illustrated by Töndury (1955). Two main types of damage were noted, the formation of giant cells, and of cellular degeneration accompanied by nuclear breakdown and the formation of deeply staining cytoplasmic lumps. The giant cells presumably arise from the suppression of cell-division followed by the formation of a restitution nucleus. These nuclei stain rather palely, and appear to be lacking in DNA. On the other hand, the cytoplasmic globules which are commonly met with appear to consist of RNA, and to indicate an over-production of that compound.

In some experiments with young amphibian embryos (Axolotl, *Triturus alpestris*, and *Xenopus*) similar cytological pictures have been seen. The embryos were exposed to solutions of TEM or of Myleran, at a concentration of 0·01 per cent. The ages at exposure ranged from blastula to early tail-bud, and most of the embryos were left in the solution until fixation some days later. The point of interest, which does not emerge clearly from previous accounts, is that one often finds a much greater degree of damage in the neural tube than in any of the other tissues. Externally such embryos are short, and appear to have a 'hollow back' In sections, one sometimes sees the neural tube very extensively abnormal, with almost every cell affected, while the remaining tissues look reasonably healthy. Figs. 1 and 2 of the Plate show two specimens from experiments with TEM, in which nearly all the neural cells exhibit cytoplasmic aggregates of RNA and some nuclear degeneration. In these experiments, the formation of giant cells with pale nuclei was rare with TEM, but was well seen following exposure to Myleran (Plate, fig. 3).

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Such tissue specificity is not always so well defined as in the specimens illustrated, but even when the damage is more widespread, it is, in these stages, always the neural system and its derivatives such as the eye which are most strongly affected. The ganglia of the head are as sensitive as the main body of the central nervous system, but the sense placodes, such as the otic and nasal invaginations, show the same resistance as the rest of the ectoderm.

The biological mode of action of these alkylating cytotoxic agents is as yet too little understood for it to be possible to discuss profitably the chemical reasons for the differences in sensitivity of different tissues. It is important to note, however, that the tissue specificities may differ widely between one organism and another. The contrast between the great susceptibility of the mesoderm in chick embryos, described by Jurand (1958), and the sensitivity of the neural system in amphibian embryos is sufficient warning that the reaction to these compounds cannot be interpreted in simple biological terms, but must be related to specific, but as yet unknown, chemical properties of the cells exposed to them. It may well be that, as Bodenstein (1954) has argued, these compounds inhibit the synthesis of DNA, but if this is so there must also be some other factors involved in their action which determine the type of cell which will be most severely affected.

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EXPLANATION OF PLATE

Fig. 1. *Triturus alpestris* larva, exposed to 0·01 per cent. TEM since late neurula stage. Neural cells degenerating but other tissues healthy.

Fig. 2. Part of the brain (on left) together with head mesenchyme (on right) from a similar embryo at higher magnification. Note darkly stained cytoplasmic accumulations of RNA in the damaged neural cells.

Fig. 3. Part of the mid-brain of a *Triturus*, mid-tail-bud stage, exposed to 0·01 per cent. Myleran since the early gastrula stage. Note the presence of some giant nuclei scattered amongst those of normal size.

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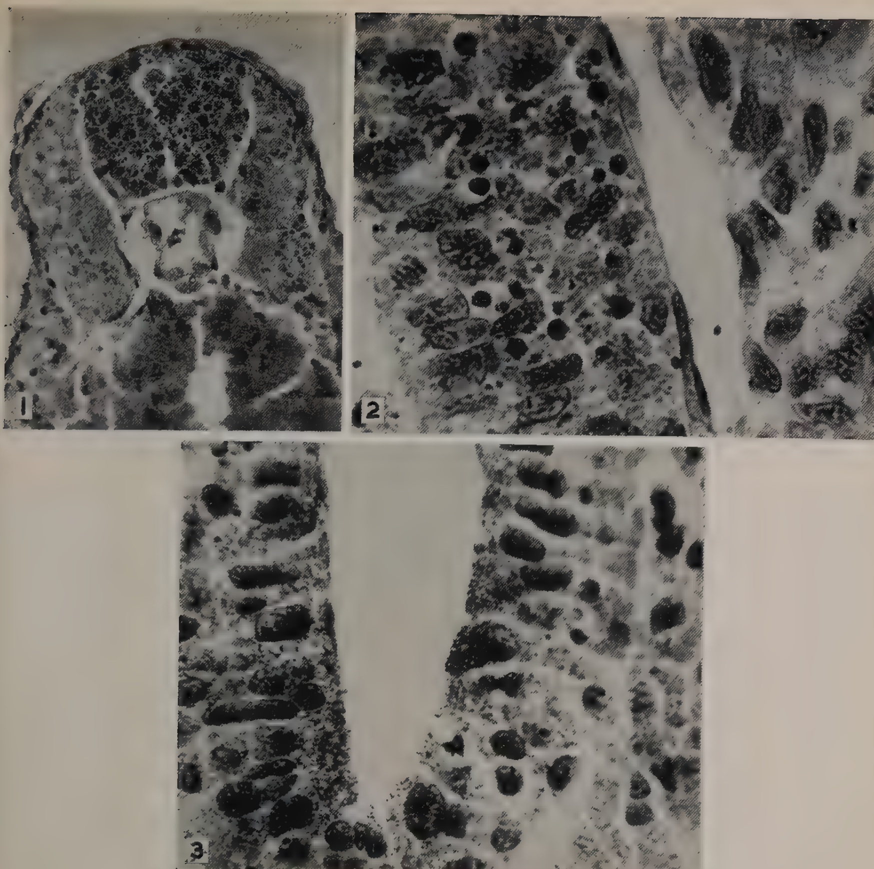

C. H. WADDINGTON

Effects of some Amino-acid and Purine Antagonists on Chick Embryos

by C. H. WADDINGTON *and* MARGARET PERRY[1]

From the Institute of Animal Genetics, Edinburgh

WITH ONE PLATE

SEVERAL authors have studied the effects on developing embryos of substances which are analogues of naturally occurring amino-acids and purines, and known to act, in other systems, as metabolic inhibitors. It was emphasized by Waddington, Feldman, & Perry (1955) that any particular substance may exhibit very different effects in embryos of different types. They found, for instance, that the purine analogue 8-azaguanine has a very strong action in the chick and a much lesser one in the newt embryo. It is therefore necessary to consider the various classes of embryos separately. In this communication we shall be concerned only with chick embryos.

Substances under test can be administered to such embryos by injection through the shell, as was done in the paper cited above. With this technique it is impossible to know how much diffusion takes place of the substance injected, and one cannot therefore be certain of the effective concentration which actually reaches the embryo. More precise results can be obtained if the substance is mixed with a medium suitable for culture of the embryos *in vitro*. The culture method introduced by New (1955), in which the embryo is left attached to the vitelline membrane, and is cultured, endoderm side upwards, on the surface of a few drops of albumen, results in the development of embryos which are normal enough to serve as controls against which the effects of substances added to the medium can be assessed. This method has therefore been used in many of the experiments to be reported here. It is, of course, considerably more time-consuming than simple injection into the shell, but the effects which have been found are sufficiently well marked to render the performance of very large series of experiments unnecessary.

The substances dealt with in this report are two amino-acid analogues, ethionine and *p*-fluoro-phenylalanine, and two purine analogues, 8-azaguanine and azahypoxanthine.

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DESCRIPTION OF EXPERIMENTAL RESULTS

Purine analogues

It has previously been reported (Waddington, Feldman, & Perry, 1955) that 8-azaguanine, injected into the shell during primitive streak and early somite stages, causes cellular degeneration of the presumptive axial mesoderm which is at the streak stage of development, of the brain vesicles, and to a somewhat lesser degree of the newly formed somites. In those experiments, the egg, whose volume is about 50 ml., had been injected with 0·5 ml. of a 0·05 per cent. solution, but it was impossible to be certain of the concentration at the blastoderm. When embryos are cultivated *in vitro* it has been found that a similar intensity of damage is produced by a concentration in the medium of 0·0025 per cent. Frair & Woodside (1956) observed damage of the same nature, though rather less intense, in a concentration only a quarter of this.

Very similar effects are produced by azahypoxanthine. This substance has been administered by injection. A moderate degree of damage, rather slighter than that described for 8-azaguanine, was caused by 0·5 ml. of a 0·1 per cent. solution. Similar injections of a 0·05 per cent. solution had quite appreciable effects, but there was very great variability between embryos. This made it impossible to determine whether any alleviation occurred when hypoxanthine was injected simultaneously. However, even when the concentration of the hypoxanthine solution was 0·5 per cent., there were still a number of abnormalities attributable to the 0·05 per cent. azahypoxanthine, so the alleviation, if any, cannot have been anywhere near complete.

In sections of the affected embryos, one can, in the less damaged regions, study the types of cellular degeneration which occur. It is characteristic of both azaguanine and azahypoxanthine that one finds many cells arrested in the early prophase of division (Plate, fig. 1). This is particularly noticeable in the cells bounding the lumen of the brain vesicles. In sections stained with methyl green-pyronin, one finds in this region many cells with large nuclei which stain diffusely with methyl green, showing a barely recognizable network of chromosomal threads. These indicate the beginning of prophase, but they take up less stain than would normally be expected at this stage. The appearances suggest that there has been an inhibition of DNA synthesis.

In other affected cells, which are not at a mitotic stage, the characteristic type of degeneration is the appearance of pycnotic nuclei, which stain deep red in pyronin. It is possible that these represent a later stage in a process which begins with the prophase arrest described above, but there are no definite indications of this, and the pycnosis may equally well be a quite distinct phenomenon.

Amino-acid analogues

(*a*) *Ethionine*. In an earlier publication (Feldman & Waddington, 1955), it was shown that the injection of ethionine into the egg causes a retardation in the

rate of differentiation and the appearance of some rather slight abnormalities. By an unfortunate error, the doses used were given wrongly in that paper. The injections actually consisted of 0·5 ml. of a 1·1 per cent. solution, which would give a concentration of $6{\cdot}7 \times 10^{-4}$ M when diluted in the egg contents (not 10^{-6} as stated).

In more recent experiments injections have also been made into later stages, the recipients being mostly incubated for 48 hours before injection. With an injection of 0·5 ml. of 2·2 per cent. solution, inhibition of growth and delay in differentiation was clearly appreciable, although the embryos were very variable, probably owing to the incomplete homogenization of the injected material throughout the egg contents. Herrmann, Konigsberg, & Curry (1955) have described the effects of a slightly smaller injection, 6 mg. as opposed to the 11 mg. employed by us. They found no marked retardation of growth in embryos less than 7 days of age, but a considerable effect in later stages. With our dose, the inhibitory effect of ethionine is certainly visible very soon after the injection is made into the 2-day-old egg. The retardations occurring in our series did not always affect the whole embryo uniformly, but in some cases produced disparities between the parts which were sufficiently well marked to amount to definite abnormalities. These were of very various kinds, so that it is impossible to describe any particular characteristic syndrome; on the whole, the inhibitory effects on these later embryos seemed to be rather stronger on the anterior structures than on the posterior.

More definite effects on differentiation have been found in younger embryos cultivated *in vitro* in the presence of ethionine. Strong action was shown in cultures containing 4·4 mg./ml., but activity was not appreciable at 0·5 mg./ml. In embryos cultured in the stronger concentration from the streak or head process stages, the whole embryonic axis was very extensively degenerated. Little axial mesoderm appeared, and somite formation was very incomplete (Plate, fig. 2). The effects were, however, more general than those of the purine analogues. Whereas the latter affect the axial mesoderm considerably more than the neural plate and notochord, all three tissues seemed about equally susceptible to ethionine; in fact the neural tissue was perhaps the hardest hit and in some specimens has almost completely failed to appear.

In sections it can be seen that the type of cellular degeneration produced by ethionine is somewhat different from that found with the purine analogues. There is no sign of the cells arrested in prophase. The damaged cells exhibit large lumps of material which stains deeply in pyronin, but these are found predominantly in the cytoplasm, and it does not seem likely that they have been derived from pycnotic nuclei. There is also an increase in the general cytoplasmic basophilia of cells which do not contain large pyronin-staining lumps.

Three series of embryos were cultured in the presence of ethionine and of glycine-C^{14}, and the uptake measured with a Geiger counter. The concentrations of ethionine were chosen to produce some inhibition, but not any massive

necrosis. In the first such series (TC 8), the embryos, after fixation and extraction in cold 10 per cent. tricholoacetic acid, were dried separately on to planchettes, but it was felt that the weighings were not sufficiently accurate and that the layer of material on the planchette was too thick. In the later series (TC 11 and TC 12) several embryos were pooled and homogenized in TCA before being spread on the planchettes. The results of the experiments are shown in Table 1. It is clear that the presence of ethionine has in all cases resulted in a lower uptake of glycine. It had previously been shown (Feldman & Waddington, 1955) that it also inhibits the uptake of methionine-S^{35}.

TABLE 1

Reduction of glycine uptake by ethionine

Series	Ethionine concentration	Glycine—C^{14} per embryo	*Counts/min./mg. (corrected for background and absorption)* Glycine	Glycine+ ethionine
	(mg./ml.)	(μc.)		
TC 8	1·1	3	25,377	10,125
TC 11	2·2	3	8,338	5,625
TC 12	2·2	0·6	2,770	1,775

(*b*) p-*Fluoro-phenylalanine*. This amino-acid analogue has been administered to embryos cultivated *in vitro* from streak or head process stages. In series at 4 mg./ml. or 2 mg./ml., development of the embryo was completely prevented; growth of the blastoderm ceased, and the area pellucida showed no signs of any embryo except for a slight thickened region in its centre. Characteristically inhibited embryos were obtained at concentrations of 0·4 or 0·2 mg./ml. These in general resembled the embryos cultivated in 4·4 mg./ml. ethionine, which were described above, but the effects on the axial tissues were perhaps more severe (Plate, figs. 3 and 4). The degeneration of the mesoderm led to the appearance of large blisters on each side of the axis in some embryos, similar to those described as resulting from azaguanine (Waddington, Feldman, & Perry, 1955). The neural tissue and notochord were also attacked, as with ethionine. Fluoro-phenylalanine differs from the latter, however, in the greater intensity of the general growth inhibition which it causes. The expansion of the blastoderm in the cultivated embryos was considerably reduced. In sections, the types of cellular degeneration found were similar to those described for ethionine; again there was no sign of the prophase inhibition which occurs with purine analogues, and the damaged cells showed large cytoplasmic lumps staining in pyronin as well as an increase in general cytoplasmic basophilia (Plate, fig. 5).

The deleterious effects of fluoro-phenylalanine (at 3·5 mg./ml.) were completely alleviated by an equimolecular quantity of phenylalanine (Plate, fig. 6).

Glycine, however, had no alleviating effect, even when added in four times equimolecular concentration.

DISCUSSION

There are, of course, several ways in which amino-acids are involved in the metabolism of tissues. This is perhaps particularly the case for the sulphur-containing acids such as methionine and ethionine. One of the processes in which they are most closely implicated is protein synthesis, and unnatural amino-acid analogues might be expected *a priori* to interfere with such syntheses. Similarly, it might be expected that purine analogues would interfere with the metabolism of nucleic acids; and this might seem likely to have secondary consequences on protein formation. Following this line of thought, one might anticipate that all amino-acid analogues, and purine analogues, would strike most heavily at any regions of the embryo in which protein metabolism is proceeding most rapidly. Only if there are embryonic tissues in which protein synthesis is not only active but specially involves certain particular amino-acids would one expect to find that the analogues of different acids produced specifically distinct types of damage through their relevance to protein metabolism, although they might do so in other ways, for instance when they are relevant also to the formation of sulphur linkages.

Evidence that ethionine is concerned with general protein synthesis can be found in the fact that it inhibits the uptake not only of methionine, as reported by Feldman & Waddington (1955) but, as the present experiments show, also of glycine. A similar effect on incorporation into adult tissues has been described by Simpson, Farber, & Tarver (1950). Since the main effect of the amino-acid analogues which do not contain sulphur would be expected to be on protein synthesis, it is relevant to inquire whether there is any evidence of specifically different local effects being produced by the various analogues which have been used on the chick embryo so far, or whether they can all be supposed to impinge on the same areas where protein formation is proceeding most rapidly.

It is worth noting that both the purine analogues and the unnatural amino-acids which have been tested are similar to the extent that they have stronger effects on the embryonic axis than on more peripheral regions of the blastoderm. Even in this major point, however, there seem to be some differences between the various compounds. Fluoro-phenylalanine causes an inhibition of the expansion of the blastoderm, which is stronger, relative to its effects on the embryo, than that found with any of the other compounds.

As regards the embryonic structures themselves, there are considerable similarities for all compounds, but probably also some differences between the purine and amino-acid analogues. The mesoderm being invaginated through the streak is very sensitive to the purine analogues and to both the amino-acid analogues dealt with here, but whereas the purines leave the notochord relatively undamaged, and are not excessively harmful to the newly appearing neural

plate, both the amino-acids attack all three axial tissues more or less equally. At a slightly later stage, the brain vesicles seem, in relation to all compounds, to be rather more sensitive than any more posterior parts of the neural system which may have progressed beyond the open-plate stage at the time of exposure.

Rothfels (1954) and Herrmann, Konigsberg, & Curry (1955) have described the effects of certain other amino-acid analogues. The former showed that analogues of certain aliphatic amino-acids, at concentrations of 0·25–1·0 mg./ml., cause failures of the segmentation of the axial mesoderm, leading to the appearance of blocks of unseparated somites. Herrmann *et al.* described very similar effects produced by certain other unnatural amino-acids, which could be regarded as analogues of leucine; and they showed that the damage was alleviated by the addition of leucine, although this was not true for the substances studied by Rothfels. In both these studies, the 'somite blocking' action was regarded as a relatively specific type of effect, characteristic of some but not all amino-acid analogues. Herrmann *et al.* contrasted it with the effect of ethionine, which in their hands was found not to exert any deleterious effect on young embryonic stages, although leading to a retardation of growth in stages later than 7 days of incubation. As pointed out above, we find that ethionine, in concentrations which were probably considerably larger than those used by these authors, has a very definite effect on explanted young embryos. Thus their comparison was based on an incomplete appreciation of the effects which ethionine may bring about. Similarly, Rothfels compared the 'somite blocking' action of her aliphatic amino-acid analogues with the damage produced by analogues of aromatic acids (β-2-thienylalanine and *p*- and *o*-chloro-phenylalanine). The latter substances caused a retardation of growth and also a number of abnormalities in various regions, which are not very fully described. As far as one can see from Rothfels' account, it appears not unreasonable to suppose that these aromatic analogues had effects of essentially the same nature as those described above for fluoro-phenylalanine, although the latter seems to have been more potent.

The question whether different amino-acid analogues produce different syndromes of effects therefore reduces to the question whether the 'somite blocking' action described for the aliphatic analogues is essentially different from the type of damage described for ethionine and the aromatic analogues. The somite blocking is a mere failure of the mesoderm to separate into distinct segments; the cells remain perfectly healthy as far as sections reveal. The effect is therefore a rather slight one when compared with the abnormalities produced by the aromatic analogues. It would therefore seem possible to suppose that stronger action of the aliphatic analogues might produce a pattern of damage indistinguishable from that of the aromatic compounds. A difference in the type of activity of these two classes of analogues may perhaps be probable, but it cannot as yet be taken as certain.

At the cellular level, no difference in the mode of action of the different amino-acid analogues has yet been detected, nor is there adequate evidence to

interpret the significance of the cytological picture or to suggest how the characteristic cytoplasmic pyronin-staining lumps arise. These, together with the general increase in basophilia in the cytoplasm, suggest that in the presence of unnatural amino-acids the cells acquire an abnormally high content of RNA. This would appear to indicate that the relations between nucleic acid and protein in the cell is by no means as simple as is sometimes supposed, but involves a delicate balance which we do not yet fully understand.

It is not surprising to find that there is a marked difference between the cytological effects of the amino-acid and purine analogues. The latter would be expected to impinge on to the nucleic acid metabolism. The evidence that they cause an arrest in prophase, with the appearance of weakly staining chromosome threads, suggests that the analogues tested have affected the DNA metabolism; whether they affect RNA equally, or more or less strongly, cannot yet be stated.

It is worth drawing attention to the fact that ethionine inhibits the incorporation of labelled glycine as well as methionine. It might have seemed likely that in a system as well supplied with yolk as the chick embryo, protein formation would be based on raw materials of relatively high molecular weight, derived by a partial hydrolysis of yolk proteins. The fact that ethionine inhibits the uptake of an amino-acid with which it is not in any way homologous suggests, however, that the embryonic proteins must be in metabolic equilibrium with free amino-acids, as has been found to be the case in other systems in which a source of supply of complex raw materials is not so obvious.

SUMMARY

1. Ethionine, at a concentration of 4·4 mg./ml., produces considerable abnormalities and growth retardation in chick embryos cultured *in vitro* on a medium of albumen.

2. Ethionine reduces the rate of incorporation not only of methionine but also of glycine. Thus protein synthesis in the chick embryo, as in some adult animals, is probably by a process which involves the free amino-acid pool, rather than from intermediates of high molecular weight derived from the yolk.

3. *p*-Fluoro-phenylalanine, at 0·2–0·4 mg./ml., causes a strong growth inhibition of the whole blastoderm, and a series of abnormalities in the embryonic axis. The effect is completely alleviated by equimolecular concentrations of phenylalanine, but not by glycine.

4. In confirmation of previous work which used injection as the method of administration, it was found that 8-azaguanine is a very active inhibitor, strong effects being produced *in vitro* by 0·025 mg./ml.

5. Azahypoxanthine at 1 mg./ml. produced similar effects. No clear-cut evidence of alleviation was found with hypoxanthine.

6. The patterns of damage produced by these various substances are described. They all attack most strongly the tissues which are in the process of differentiating from the streak, and later, rather less strongly, the neural tissue and

particularly the brain vesicles. At the streak stage of development, the purine analogues affect the somite mesoderm more strongly than the notochord or neural plate, while the amino-acid analogues seem to affect all three types of tissue more equally. Fluoro-phenylalanine has a particularly powerful inhibitory influence on the expansion of the blastoderm.

7. These effects are compared with the damage produced by some aliphatic amino-acid analogues studied by Rothfels (1954) and Herrmann *et al.* (1955). It is considered that the comparatively weak effects described by these authors make it unsafe to conclude that there is any essential difference in the mode of action of the aliphatic and aromatic analogues. Both may be acting simply on sites of rapid general protein synthesis.

8. At the cytological level, there is a clear difference in the type of damage caused by the purine analogues and the amino-acid analogues studied. The former cause an arrest of mitosis at early prophase, with the appearance of weakly staining chromosome threads; in other cells the nuclei become pycnotic, and stain deeply in pyronin. Prophase arrest is not found with amino-acid analogues, and the pyronin staining lumps found in degenerating cells are characteristically located in the cytoplasm, which also shows an increase in general basophilia.

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EXPLANATION OF PLATE

Fig. 1. Brain region of embryo cultivated in 8-azaguanine (0·0025 per cent.). Note many arrested mitoses bordering the lumen. Methyl-green-pyronin.

Fig. 2. TC 6. Embryo cultivated from streak stage in ethionine (0·44 per cent.).

Fig. 3. FPA-5. Embryo cultivated from streak-stage in *p*-fluoro-phenylalanine (0·4 per cent.).

Fig. 4. FPA-7. Embryo cultivated in same conditions as fig. 3. The darkly stained region at the head is the inhibited and contracted vascular area of the blastoderm.

Fig. 5. TC 14. Section of brain embryo cultivated in *p*-fluoro-phenylalanine (0·4 per cent.). High cytoplasmic basophilia and pycnotic nuclei. Methyl green-pyronin.

Fig. 6. Embryo cultivated as in figs. 3 and 4, but with the addition of an equimolecular concentration of phenylalanine. Almost complete restoration of normality.

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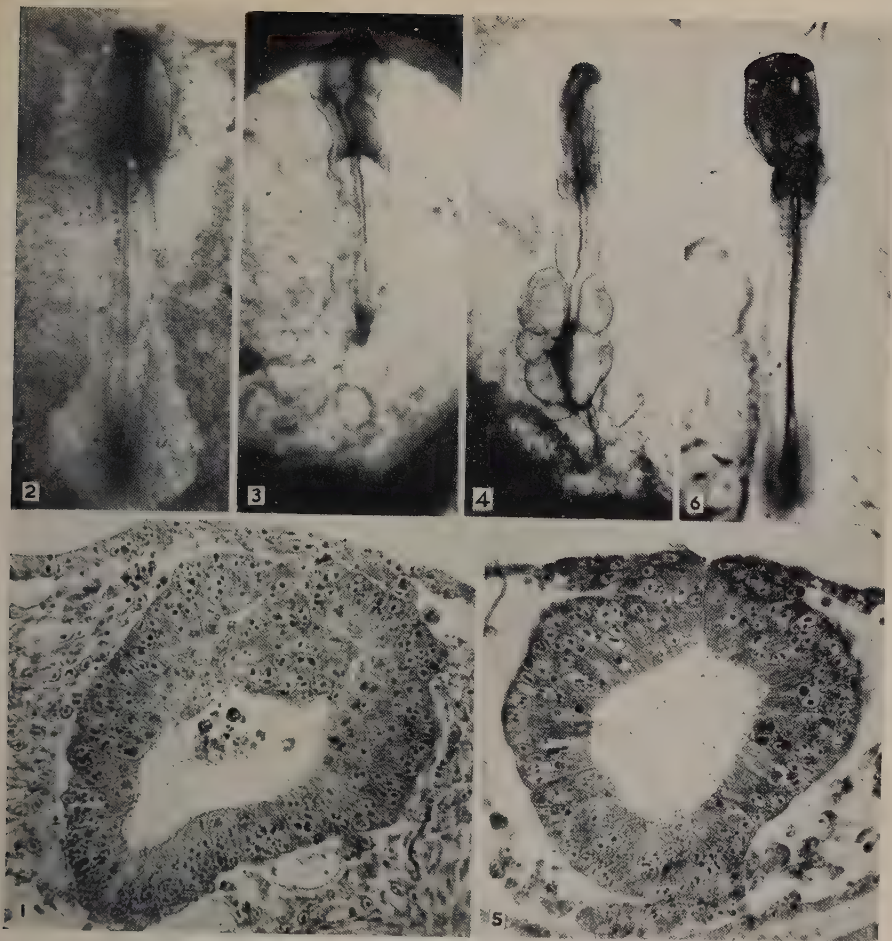

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